DOCTORAL THESIS

PATTERNS OF PARASITE INFECTION IN THE AFRICAN ELEPHANT POPULATIONS IN KENYA

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BACKGROUND

1. BACKGROUND

1.1. African elephant Biology and Conservation status

The African elephant is the largest land mammal. The word 'elephant' translates into 'huge arch', which comes from viewing the elephant from its side. The etymology of the word 'elephant' is from *ele*, a Greek derivative meaning 'arch' and *phant*, a Greek derivative meaning 'fantastic. The African elephant's scientific name, *Loxodonta africana* was coined in 1827, whereby *Loxodonta* describes the lozenge pattern of the enamel on the chewing surface of the tooth and the species name *africana* denotes it's origin and habitat (Aguirre, 1969; Gheerbrant, 1998).

The African elephant is a flagship species that faces multiple and diverse conservation challenges. Both the elephant ranges and demography have continued to decline due to various anthropogenic forces. Currently, the African elephant is listed as a threatened species, specifically due to poaching, which has decimated some populations and contributed to significant decline in demography. Moreover, changes in land-use across the African ranges, has resulted in small disconnected elephant sub-populations (Chase *et al.*, 2016). The convergence of these factors enhances vulnerability of threatened species, especially to impacts of disease (Patz *et al.*, 2000). Pathogens and parasites that infect elephants are well described, except there is little information on how the parasites interact with the emerging conservation strategies and changing abiotic factors, such as climate and host population isolation.

The African elephant and its closest relative, the Asian elephant (*Elephas maximus*) are the only surviving species of the order probocidea. These two genera originated in sub-saharan Africa in the early Pleistocene (Whitmore, 1977), *Loxodonta* remained in Africa while *Elephas* moved to Asia during the late Pleistocene. There are two known subspecies of the African elephant: the savannah elephant, *Loxodonta africana*

(Blumenbach, 1797), and the forest elephant, *Loxodanta cyclotis* (Matschie, 1900; Lausen & Bekoff, 1978; Barriel *et al*., 1999; Grubb *et al.,* 2000; Groves & Grubb, 2000; Debruyne, 2005).

The savannah elephant is larger than the forest elephant, with sparser body hair, and more triangular ears that are larger and thicker, it has curved tusks unlike the straighter, narrower downward pointing tusks of the forest elephant (Lausen & Bekoff, 1978). As postulated by Western, 1986, the elephant intermediate between the two subspecies are found in hybridization zones in Africa where forest and savannah ecosystems merge.

African elephants live in a fluid, fission-fusion society system in which males and females live in separate but overlapping sphere (Douglas-Hamilton, 1972; Martin, 1978; Moss, 1981; Moss & Poole, 1983; Hall-Martin, 1987; Poole, 1994; Sukumar, 2003; Wittemyer *et al*., 2005). These societies are thought to allow individuals to respond to changes or threats by constantly changing the number and identity of animals that they associate with (Archie & Chiyo, 2012).

1.1.1. The elephant Society – Female unit

Female elephant society consists of complex relationships extending from mother-offspring bond to family units, bond groups and clans (Douglas-Hamilton, 1972; Martin, 1978; Moss, 1981; Moss & Poole, 1983; Hall-Martin, 1987). The family is the basic social unit for elephants, it is usually composed of one to several related females and their young calves may vary in number from two to thirty individuals (Moss, 1988). Being part of a large family is beneficial for the elephants, for example, related females will form a defensive unit againist any perceived threat and form coalition againist none related females or males (Moss, 1988).

According to Moss (1988), larger families comprised of older matriarchs, who are the knowledge repositories (McComb *et al*., 2001), can dominate smaller families with younger

matriarchs, thereby enabling them to compete more successfully for the scarce resources. Indeed, the larger the family, the better the chances of calf survival, because a large family has more females to tend to the calves (Lee, 1987; Lee, 1989).

Elephants may also form bond groups (Moss, 1981; Moss & Poole, 1983) which are a combination of several closely related families (Moss, 1988). Bond groups appear to be formed when family units become too large and split along family lines (Moss, 1988). Members of a family or bond group are usually related (Moss, 1981; Moss & Poole, 1983) and they show a unique greeting ceremony (Moss, 1981; Moss, 1988), high rate of association over time and act in a coordinated manner, largely, they show affliative behavior towards each other. Elephant society may also be described in terms of a clan, which is a level above the bond group (Moss, 1981) and is defined as families and bond groups that utilize similar home range especially during times of resource scarcity (Moss, 1981; Moss & Poole, 1983). However, clan is not a very clear concept for describing elephant's social units.

It is very unusual for a female elephant to be found alone. Rather, it can be found in a number of different types of groups, either in a segment or in fragment of a family unit or in an aggregation of elephants numbering in thousands comprising several clans (Moss, 1981; Moss & Poole, 1983; Lee, 1987). The size and composition of a group in which a female elephant finds herself in may change hourly, over some days or within a season (Moss & Poole, 1983; Wittemyer *et al*., 2005). This depends on the size of her family, the number of individuals that make up her bond group, her sexual state, the strength of bonds between her and other family members, the habitat, the season and mostly, the level of human threat (Moss, 1981; Moss, 1988; Poole & Moss, 1989; Kangwana, 1993; Njumbi, 1993). In times of abundance of resources, elephants are known to aggregate (Western & Lindsay, 1984; Moss, 1988; Poole *et al*., 1988) and when the resources are scarce, the aggregation and bond groups begin to split up (Moss, 1977; Moss, 1981; Moss, 1988). Moreover, in

times of prolonged droughts, even family units may break up for prolonged periods (Moss, 1977; Moss, 1981; Moss, 1988). Elephants that inhabit savanna are known to aggregate in response to human hostility (Kangwana, 1993). These aggregations are unique and differ from the social aggregations by the tight bunching pattern of elephants (Moss, 1988) equally so, aggregation can be in response to poaching threat (Laws *et al*., 1975; Eltringham, 1977; Eltringham & Malpas, 1980; Abe, 1982; Poole, 1989c; Njumbi, 1993).

Family units within tropical forests are smaller (Merz, 1986; White *et al*., 1993), compared to those that inhabit a more open savanna grassland (White *et al*., 1993). The elephant is a mammal with long lifespan, and their age determines dominance, leadership and calf survival among females (Moss, 1988), and reproductive success through female choice and dominance among males (Poole, 1989a&b). Older matriarchs (> 35 years old females) lead larger families than their younger counter parts (Wittemyer *et al*., 2005). The individual members in a family unit constantly observe and imitate the actions of the older matriarch which facilitates learning (McComb *et al*., 2001).

Females in the same family move in a coordinated pattern, have affiliative behavior towards each other, and consistently associate with each other (Moss & Poole, 1983). Like many social mammals, female elephants are matrilocal (Figure 1.1.), whereby females remain with their relatives; therefore, families are often composed of close maternal kin (Archie *et al*., 2006). Although both male and female elephants utilize specific home ranges at specific times of the year, it should be noted that none of the two genders are territorial (Moss & Poole, 1983; Hall-Martin, 1987).

Figure 1.1. A family of elephants in Shaba National Reserve within Laikipia Samburu Ecosystem.

1.1.2. The elephant society - Male unit

Male elephants are known to disperse from their natal groups as teenagers (Poole & Moss, 1981) and often assemble in bachelor groups (Eisenberg *et al*., 1971; Poole & Moss, 1981), in which, they frequently spar to assess their status (Moss, 1983; Chiyo *et al*., 2011). All male association may be motivated by the opportunities available for learning social skills by the younger males from the older more experienced males (Evans & Harris, 2008; Chiyo *et al*., 2011). Moreover, elephant males have complex relationships, where they prefer to associate with age-peers in the case of sexually inactive males (Chiyo *et al.,* 2011, Goldenberg *et al*., 2014) and related males (Chiyo *et al*., 2011). There is some evidence for older males being preferred associates or being more central to male society than young males (Evans and Harris 2008, Chiyo *et al*., 2011, Murphy *et al*., 2020). Male associations were also shown to facilitate social learning, for example: bulls who had an older crop raider as a top associate were more likely to raid themselves (Chiyo *et al*., 2012). Thus, tem porary all-male groups seem to not only provide an opportunity to spar and test strengths, but also possibly for younger males to learn from knowledgeable, older males (Chiyo *et al*., 2012).

Physiologically, male elephants reproduce at 14 - 17 years; this therefore gives them a reproductive lifespan of above 40

years, although the more mature counterparts competitively exclude younger males from active reproduction (Rasmussen, 2005; Hollister-Smith *et al.,* 2007; Rasmussen *et al*., 2008).

Males continue to grow in height and weight throughout most of their lives, eventually reaching almost twice the weight of adult females (Law, 1967; Hanks 1969). Due to the early rapid growth in males, combined with a higher rate of growth throughout their lives, this leads to a higher degree of sexual dimorphism observed in adults (Lee & Moss, 1986; Lee & Moss, 1995). Most mammals cease to grow once they attain the age of sexual maturity, this, however is not true for elephants (Haynes, 1991). They continue to grow even after attaining sexual maturity; this is so because of the unusual delayed fusion of long bones, which is more pronounced in males than in females (Haynes, 1991).

In female elephants, fusion of the long bone occurs between 15 and 25 years of age while in males it occurs at between 30 and 45 years of age (Haynes, 1991). This effectively make the male elephant structurally large bodied than their female counterparts. Males mature later than females and although they start sperm production at around 14 years of age (Laws & Parker, 1968), they are not yet socially mature and do not compete with older males for oestrus females until they are in their early 20's or late teens (Poole, 1989a). Naturally, males will not be able to father their first offspring until they are between 30 and 35 years old, at about 45 years they reach their prime (Poole, 1989a&b).

Males above 25 years of age begin displaying periods of heightened sexual and aggressive activity termed as musth (Poole & Moss, 1981; Ganswindt *et al*., 2005; Rasmussen, 2005). Indeed, males in such state cover larger areas at a faster pace compared to their non-musth counterparts (Taylor *et al*., 2019). Musth is such a powerful tool in the male elephant world that it can temporarily raise an individual's dominance above that of the non-musth males (Poole, 1987; Poole,1989a).

Musth manifestation includes; a sharp rise in aggressive behavior, copious secretions from enlarged temporal glands and

continuous discharge of urine (Poole & Moss, 1981; Poole, 1982). The older the male the more regular and prolonged periods of musth they encounter within a year, younger bulls have less regular periods of musth that relatively last shorter (Poole, 1987; Poole *et al*., 2011).

Figure 1.2. High-ranking bull leading his counterpart on a crop-raiding mission

If males are not in musth, their dominance is determined by age and body size (Figure 1.2.), where larger, older males rank above smaller younger males (Poole, 1989a). Once in aggressive state of musth, a male ranks above all non-musth males (Poole, 1989a). When in musth, male dominance is determined by a combination of body size and musth condition, if two males are in musth and are closely matched, they will often fight, mostly to the death of one of them (Hall-Martin, 1987; Poole, 1989a). Therefore, it means that the reproductive ability of a male elephant increases with age, this is intensified by the size and dominance of these individuals at this advanced age and the preference of females for such individuals (Moss, 1983; Poole, 1989b).

1.1.3. Elephant sexual maturity and behavior

Individual males, locate, guard and mate with as many oestrus females as possible during their musth periods. Musth males are more successful at obtaining mating females than their non-musth counterparts because of their large body size and their aggressive behavior (Moss, 1983; Poole, 1989b). Moreover, females will often prefer to mate with musth males, indeed they will not stand for a younger non-musth male, and if approached by such a male, they will solicite-guarding behavior from a musth male (Moss, 1983; Poole, 1989b). In normal cases, males will reach their prime at 45 years of age and will probably father their first offspring at between 30 and 35 years (Poole, 1989 a&b).

Nevertheless, younger males still contribute to the gene pool despite the obvious reproductive dominance by the older bulls (Hollister-Smith *et al*., 2007; Rasmussen *et al*., 2008). Therefore, competition for females may also exist among non musth males, and male-male affiliative associations are expected to be weak. Older males are also vital repositories of ecological information and fitness as well as social information that younger males rely on (Evans & Harris, 2008), a similar role-played by matriarchs in a breeding herd (McComb *et al*., 2001).

Behavioral studies show that oestrus lasts between four and six days (Moss, 1983; Poole, 1989b; Mutinda, 1995). Studies have shown that ovulation and conception occur at the mid-oestrus when females are guarded and mated by a high-ranking musth male (Poole, 1989a; Mutinda, 1995). A female in oestrus will attract males by exhibiting conspicuous behavior, (Moss, 1983; Mutinda, 1995) loud frequent calls (Poole *et al*., 1988; Poole, 1989b) and producing urine with particular olfactory components (Rasmussen, *et al*., 1982; Mutinda, 1995). Females that fail to conceive during this period will come into oestrus again in three months' time. Most elephants do not exhibit a pronounced breeding season (Hall-Martin, 1987; Poole, 1987), though the occurrence of oestrus and conception is sensitive to

resource availability and rainfall (Laws & Parker, 1968; Laws, 1969). Therefore, habitat type and rainfall are factors that determine seasonality degree of oestrus (Laws & Parker, 1968; Hanks 1969; Laws, 1969; Hall-Martin, 1987; Poole, 1987).

A long-term study of elephants in Amboseli revealed that the frequency of oestrus is significantly higher during and following the wet seasons (Poole, 1987; Moss, 1988) when the females are in good condition. Equally so, the oldest, highest ranking males come into musth during and following the rains when food is in plenty and most females are in oestrus (Poole, 1987).

Both sexes of elephants show their reproductive readiness through postural, auditory, and chemical signals. Studies have shown that males do not have to be in musth to mate, although oestrus female elephant's prefer musth males (Poole, 1989b).
Musth males portray a unique posture, and aggressive intentions are very clear due to his raised head, extended ears, and forward motion (McKay, 1973). According to Moss, (1983) behaviors that shows a female African elephant in oestrus, includes enhanced wariness, spatial separation from the herd, leading males about at a walk or chase, and keeping company of a single male for several days. Oestrus females may advertise their reproductive status to attract males using infrasonic vocalizations which is audible over many kilometers, (Pool*e et al*., 1988; Langbauer, *et al*., 1991). Vocalization by females may also occur following a mating (Pool*e et al*., 1988; Langbauer, *et al*., 1991). When males join a female herd, they promptly inspect, fresh feces, urine deposits and the genitals of each female, including very young ones (Poole & Moss, 1981; Poole, 1982). There is a display of a flehmen response by males to females approaching ovulation. In this case, the trunk tip transfers material from the source to the ductal openings of the vomeronasal organ in the roof of the mouth (Rasmussen, *et al*., 1982; Mutinda,1995). A compound in the urine of Asian elephants was identified as an oestrus pheromone that the males are keen on detecting (Rasmussen *et al*., 1996).

During ovulation period, the concentration of this pheromone is higher and gradually reduces as when the ovulation period is over (Rasmussen *et al*., 1996). Indeed, African elephant males show similar behavior as their Asian counterparts of urine inspection, although the African female elephants do not possess a similar pheromone in their urine (Hall-Martin, 1987; Poole & Moss, 1989).

While mating, the female stands still while the male mounts her and copulates; it is a rather short act. More often than not, the male uses his trunk to hold and properly position the female for mating to occur (Poole, 1989a; Mutinda, 1995). Males at all levels constantly practice the act of mounting, maybe as a sign of dominance or as a learning process (Hall-Martin, 1987; Poole, 1989a).

During such times of resource abundance, females aggregate into large groups, increasing the probability that a male will find a female in oestrus (Poole & Moss, 1989). Elephants are born after a gestation period of 21.5 months with an average birth weight of 1,320 kg for males, 20-30 kg more than that for females. Sex ratio of births is 50:50 though there is evidence that slightly more males are conceived during years of higher than average rainfall (Moss, 1988). Calves born to older larger females are bigger than those born to younger, smaller females (Lee, 1986). According to Laws & Parker (1968), twinning occurs in less than 1% of all conceptions. For the first 3 months of their lives, the energy requirements of calves are met by exclusively suckling from their mothers. Thereafter, calves start feeding independently with the time spent feeding increasing rapidly between four and 24 months when it levels up to about 55% of the daily time (Lee & Moss, 1986). Most calves suckle until the birth of the next calf, but some are weaned just before the birth of the next calf, however, some continue suckling even after the birth of their sibling (Lee & Moss, 1986).

Within the elephant family, calves are taken care of by family members and siblings, juveniles and adolescent females often comfort, assist and protect calves (Lee, 1987). These

females are described as allomothers (Lee, 1987). Allomothers are family members that included non-sibling relatives (Lee, 1987). This close care taking relationships helps bond and stabilize the family units (Lee, 1987). Allomothering is believed to increase the calf's chances of survival and possibility for adoption among other aspects (Douglas-Hamilton, 1972). Calving interval is spaced between approximately 3 years and 9 years, with individuals that are nutritionally stressed or living in a high-density population exhibiting longer calving intervals (Laws & Parker, 1968; Laws *et al*., 1975; Eltringham, 1977). Females between the age of 14 and 45 years have the highest fertility rate with means interbirth intervals increasing to 5 years by the age of 52 and six years by the age of 60 (Moss, 2001). There is heavy reliance of interbirths on habitat condition and the population density, with some interbirth intervals of up to 13 years (Laws, 1969).

Calf survival is solely depedent on the mother, for example, younger and older mothers are at a higher risk of losing their calves than the middle-aged mothers (Moss, 2001). Generally, calf mortality is at its highest in the first 12 months of the calf's life, there after the mortality is low (Lee & Moss, 1986). The mothers rank within the family unit; her experience and her general body condition are all important factors in the survival of a calf (Moss, 2001).

1.1.4. Elephant Ecology

Environmental factors affect elephant population dynamics, migration patterns, diet, group size, home range and group composition (Osborn, 2004; Ngene, *et al*., 2017). The area over which an elephant travels depends on resource availability, i.e. water, food and mates (Osborn, 2004; Ngene, *et al*., 2017). Since these resources vary seasonally, so does the elephant movements (Western & Lindsay, 1984; Baskaran, *et al*., 1995; Osborn, 2004;). Predation and human pressure greatly influence elephant

movement as well (Western & Lindsay, 1984; Baskaran, *et al*., 1995). Diets of elephants may include; grass, tree barks, herbs, fruits and tree foliage. Elephants that mainly inhabit the savanna have their diet composed of up to 70% grass during the wet seasons and a large proportion of browse during the dry seasons (Douglas-Hamilton, 1972). Unlike Savanna elephants, fruits constitute a major component of forest elephant's diet (White *et al*., 1993). Other than humans, perhaps elephants are the only animals capable of greatly affecting vegetation structure of an area thereby altering the animal community within such areas (Laws, 1970; Cumming, 1982; Western, 1989). Elephant's at high densities have been known to reduce woodlands into open grasslands (Laws, 1970; *Laws et al*., 1974; Cumming, 1982; Western, 1989).

In many elephant range states, habitat loss due to human population growth and poaching has interfered with elephants migratory routes and forced them to concentrate almost entirely within pockets of protected areas (Poole,1987; Western, 1989). Elephants are vital as agents of seed dispersal; this is an important ecological role, some plants depend on for their dispersal and survival (Alexandre, 1978; Hawthorne & Parren, 2000). According to Hawthorne & Parren (2000), elephants have played a significant role in shaping West African rain forest vegetation. This in turn shapes the mammalian inhabitats of such ecosystems (Western, 1989), because they are keystone species, elephants are very important in maintaining connections in the food web and their removal from an ecosystem may prompt a downward trend of change spanning through the mammalian habitats and the vegetation thereof (Western, 1989). The diversity of forests and savanna is greatly attributed to the elephant inhabitants (Western, 1989).

1.2. Gastrointestinal helminths in the African elephant

Due to their keystone status, elephants continued existence is very important to ecosystem integrity and the survival of other species that share habitats with them. Therefore, disease management within free ranging elephant populations ultimately translates to elephant conservation and preservation. Nevertheless, infections and diseases in elephants are understudied. Parasites of elephants are a rarely studied subject, in fact, with the existing information mostly focused on taxonomy rather than epidemiology (Condy,1974; Kinsella *et al*., 2004; Carreno & Kinsella, 2008). Disease outbreaks, especially parasitic diseases, have negative consequences on animal populations including wild elephants (Funk *et al*., 2001). The attitude towards parasitic infections is negative, mostly because it is widely thought that they cannot lead to species extinction (MacPhee & Flemming, 1999).

Most wild animals have endemic stability, whereby the parasites and the host immune system strikes a balance, this means that unless there is a shift in the balance due to factors such as injuries (Fowler and Mikota, 2006; King'ori *et al*., 2018), then there is no clinical disease manifestation (Apio *et al*., 2006). Because clinical disease is not always evident, this has led to the neglect of parasitic infections in wildlife (Gunn & Irvine, 2003). Precisely, helminths and their hosts coexist such that the development of clinical illness is rare (Fowler & Mikota, 2006). Other than injury, (whether anthropogenic or otherwise), other factors such as drought, lactation, concurrent infections and pregnancy might tip the host- parasite equilibrium leading to a diseased state (Grossman, 1985; Schalk & Forbes,1997; Vidya & Sukumar, 2002). Parasite transmission dynamics in elephants is partly influenced by their complex social structure (Altizer *et al*., 2003; Ezenwa, 2004).

Helminths are diverse organisms that parasitize gastrointestinal parasites of several mammals, including African

elephants. Previously, Obanda *et al.,* (2011), documented parasite-associated deaths in malnourished juvenile elephants in Kenya, which highlight the importance of parasitism on population dynamics of elephants. The nematodes and trematodes are the two groups of helminths common in African elephants and their transmission is environmentally dependent. The genera *Murshidia, Quilonia* and *Khalilia* are the most common nematode species infecting African elephants (Van Der Westhuysen, 1938). Potential factors determining the transmission of parasites include environmental conditions (Morgan *et al.*, 2012; Pfukenyi *et al.*, 2013) that affect the viability and behavior of parasites. The internal conditions in the host e.g. gut physiology and the immune system response affects the success and reproduction of the parasites (Sukumar, 2003). It is therefore important to study the relations between parasite loads and the body condition.

1.2.1. The influence of social structure and environmental factors on helminth infection patterns in the elephant.

The free-living environmental stages of gastrointestinal nematodes are strongly affected by climate. Extreme temperatures are detrimental to development and survival, while moisture is needed for development and translation of larvae from soil to pasture, and so rainfall and vegetation may be limiting factors for their transmission and may influence patterns of inter-population variability in infection patterns (Morgan *et al.*, 2012). In support, some studies in Africa have found a significant positive correlation between mean annual precipitation (rainfall and relative humidity) and nematode infection rates of animals (Pandey *et al.*, 1993; Morgan *et al.*, 2012; Pfukenyi *et al.*, 2013).

Several authors (Apio *et al*., 2006; Fowler & Mikota, 2006; Froeschke *et al.*, 2010) have found associations between precipitation and different qualitative measurements of egg

burden (mean nematode species richness, mean number of nematode worms and infection intensity per individual host). In one of the most recent studies, Baines *et al* (2015) demonstrated that nematode eggs (mainly strongyle type morphologically) were more common in larger groups and nematode egg counts were significantly higher in elephants living in matriarchal groups than in bachelor herds. Fluke egg morphology was similar to *Protofasciola robusta* an intestinal fluke that was associated with emaciation and deaths of elephants in Kenya (Obanda *et al*., 2011). Fluke egg prevalence increased with increasing elephant age (Baines *et al*., 2015). Further, Baines et al, (2015) found that factors such as sex, age, group size and composition and the month of the year were linked to parasite presence and the level of infection. Equally so, Thurber *et al* 2011, found that group members from family units had significantly higher fecal egg counts than male elephants. Within the family unit members, strongyle egg counts were higher for older individuals while within the male society, the younger you were the less the infection burden. Following times of higher rainfall, the infection burden was low (possibly, due to resource abundance) (Arneburg *et al*., 1998). More over dry periods come with low water supply, leading to crowding of individuals and families at the few available watering points, hence predisposing them to parasites (Krecek *et al*., 1989; Masangane *et al*., 2004). Such a depiction is shown by a decrease in the numbers of strongyle eggs within the bull society; indeed, the hormonal state (musth or non-musth) of the bull had no effect on the infection burden. As indicated by Poole & Moss (1981), bulls in musth rarely associate with other bull and often roam alone in search of oestrus females; as such, they are in low contact with other bulls that in turn lowers their exposure to parasites.

Parker *et al*., (2020) indicated that several factors played a role in determining the level of strongyle infection in wild African elephants. These factors were divided into, individual characteristics, environmental factors and social factors. Younger animals were found to have higher strongylid loads than older

animals, probably due to their under-developed immunity (Albery *et al*., 2018). The ageing of elephants in this study was more precise than previous studies by Baines *et al*., 2015 and Thurber *et al*., 2011 that did age class estimate. However, Parker *et al*., (2020) only focused on elephants aged up to 21 years for the female and up to 18 years for the male, effectively leaving out the aged animals whose parasite infection dynamics would have been of interest.

On the influence of sex, Parker *et al*. (2020) concluded that bulls have lower strongylid egg count than females and calves, which is a consistent pattern as reported by Thurber *et al*., (2011). This pattern was attributed to the male dispersal behavior and ultimately spending less time with the family groups (Evans and Harris, 2008). Male elephants spend more time in less crowded habitats away from families (Moss, 1988), which accords them parasite evading advantage as such feeding areas are considered less contaminated by dung that mainly acts as a larva reservoir (Condy, 1974). The more pronounced the male dispersal is the less the chances of infection from strongylids. Further, Parker *et al* (2020) realized that elephants that spent more time outside protected areas had higher strongyle loads, which could be due to difference in habitat parameters (Condy, 1974). The obvious differences in vegetation type within, and without the protected areas, and the differences in soil type could enhancing growth of infective larvae. Condy, (1974) indicated that the infective larvae can survive better in soils with small particles for up to three months. Thus, Burma (2015), suggests that the change in land use and the increased land utilization may have significantly accentuated wildlife diseases (Buma, 2015). It is also perceived that the constant human elephant interactions often end up with harm being meted on the elephants, the physical and often psychological harm on elephants not only does it alter their normal behavior (Wittemyer *et al*., 2019) but lowers their immunity, hence, the higher strongyle loads in elephants that are frequently outside protected areas (Parker *et. al*., 2020).

In Chad Basin National Park located in the semiarid area of northeastern Nigeria, Mbaya *et al.* (2013) studied intrinsic (host demography) and extrinsic (environment and population density) factors that might affect prevalence and worm burden of gastrointestinal parasites in African elephants. They analyzed the effects of demography (sex and age), rainfall and season on the worm burden of gastrointestinal parasites on free-living African elephants. The Chad Basin National Park has large concentrations of free-living elephants that constantly share pastures with the nomadic cattle. This study realized that gastrointestinal parasites infection prevalence was higher in bulls than the females and younger calves had more infections than the females. These findings were in contradiction with the study by Thurber *et al* (2011) in Etosha National Park Namibia where the average number of parasite burden (eggs per gram) was significantly higher in family group members than in bulls. Younger animals are more susceptible to infections probably due to their naïve immune system while adults that are already repeatedly predisposed to infections are resistant (Soulsby, 1982).

According to Mbaya *et al* (2013), higher infections in bulls is that after their dispersal (mostly as teenagers) from matriarchal family groups, bulls' form loose associations with other bulls (Poole, 1989b) and they constantly visit different matriarchal groups in search of oestrus females. This process involves inspection of female's urine from the ground using the trunk that he eventually puts in his mouth for the "Jacobs organ" to pick up pheromones. Perhaps such activity might enhance chances of contamination of the trunk with helminth eggs or infective larvae. Further, as suggested by Mbaya *et al* (2013), during musth, bull may not feed or drink well; they are in a constant state of aggression without much of a rest, this probably creates a negative energy balance (Poole, 1999) leading to stress which has been shown to compromise the immune system leading to high parasite infection (Mbaya *et al*., 2008).

The rains in Chad Basin National Park greatly increased worm burden among elephants (Mbaya *et al*., 2013), this is contrary to the common thinking that semi-arid ecosystems rarely supports development of pre-parasitic stages of helminths due to usually high ambient temperatures (more than 40°C) (Mbaya *et al*., 2006; Bryant & Hallem, 2018).

1.2.2. Life cycle of Helminths infecting the elephants

Little is known of the life cycles of elephant strongyles. Presumably the cycles are similar to those of the strongyles of domestic livestock (Figure 1.3.) (Bowman, 2003; Miller, *et al*., 2015). Adults are found in the stomach, small intestine, cecum, and large intestine, depending on the species. Females produce fertilized eggs containing embryos in the morula stage. The eggs continue embryonating in the feces to L-1, still encased in the egg case. Under ideal conditions, hatching occurs in 1 to 2 days. The free-living livestock (Figure 1.3.) L-1 larvae feed on microorganisms in the feces and molt to L-2 and again to L-3, which is the infective stage, taking 4 to 6 days. L-3 larvae migrate out of the feces in about 1 week and climb onto vegetation. The life cycle is direct, and the elephant ingests plants or grass containing infective L-3 larvae, which mature through L-4 and L-5 livestock (Figure 1.3.) to become adults in the stomach or the intestine (Fowler & Mikota, 2006).

Indeed, gastrointestinal parasites, especially nematodes (Figure 1.3.) can reduce voluntary food uptake and drastically reduce efficiency of food utilization (Fowler & Mikota, 2006). Food absorption and retention is greatly affected by parasites thereby compromising the host's mineral and nutrient uptake. Ultimately, it is postulated that high strongyle egg burden would result in poor condition of the host animal including elephants (Fowler & Mikota, 2006).

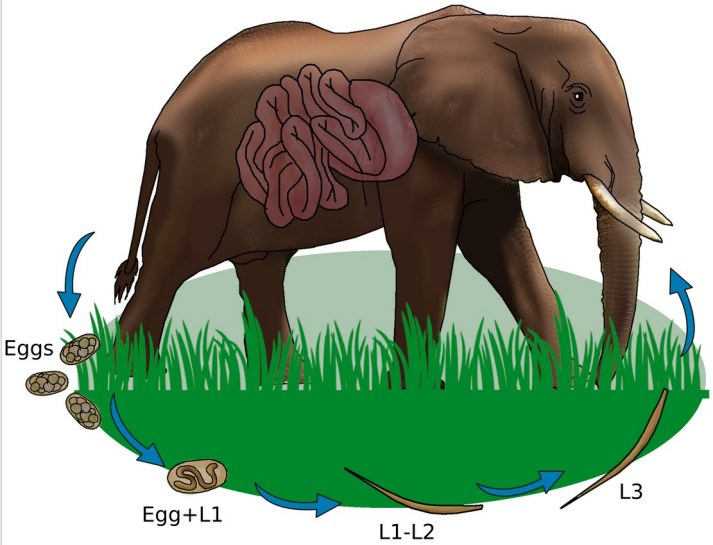


Figure 1.3. The life cycle of the strongyle parasites (Fowler and Mikota, 2006).

Several species of trematodes are known to infect elephants (Fowler & Mikota, 2006) in which some are associated with pathological lesions (Fowler & Mikota, 2006; Obanda *et al.*, 2011).

Trematodes of Fasciolocidae Family usually have a complex lifecycle that involves a host (vertebrate) where they sexually reproduce and an intermediate host (snail) where asexual reproduction occurs. The transmission of flukes is largely driven by presence of water and snails suggesting that water availability and precipitation are important in their life-history (Sherrard-Smith *et al.*, 2013). Habitat loss and fragmentation are the primary causes of biodiversity loss. Additionally, there is recognition of disease as a threat to biological diversity. Yet, few studies examine interactions between the threats of habitat loss

and disease or the links between ecosystem and animal health (Patz *et al*., 2000). Indeed, even basic baseline data on pathogens and parasites is lacking in fact, very little is known about their identity, occurrence, importance, life cycles and transmission dynamics in most wild animals, including elephants (Baines, *et al*., 2015).

According to Sukumar (2003), high parasite loads negatively affect the body condition of elephants. Naturally, intestinal parasites compete with hosts for nutrients within the gut and chances are that the parasites are better at getting most of the nutrients, thereby denying the host the much needed nutrition (Fowler & Mikota, 2006). Nematodes of the family Strongylidea that are subdivided into six genera: *Choniangium*, *Decrusia*, *Equinurbia*, *Khalilia*, *Murshidia* and *Quilonia* (Thurber *et al*., 2011; Mclean *et al*., 2012) have been found within the gut of elephants. The overall impact of infections such as those posed by strongyles is that individual health and reproduction is affected which translates into a bigger effect of shaping the entire population dynamics if several individuals are affected (Mclean *et al*., 2012). There is always the potential of strongyle infections affecting fecundity of elephants thereby altering the population dynamics and trends (Fowler & Mikota, 2006). Host immune system, seasonality and group living are factors that greatly influence parasite infection rates and severity (Turner & Getz, 2010). The biggest challenge in studying elephant strongyle infections is the ability to distinguish strongyle parasites using eggs from collected dung samples (Mclean *et al*., 2012). Nevertheless, as demonstrated, by Mclean *et al* (2012) genetic profiling of elephant strongyle parasites is possible.

Because of the association of climatic factors with propagation and transmission of both nematodes and trematodes, Normalized Difference Vegetation Index (NDVI) can be used as an indice for precipitation. Generally, NDVI is strongly correlated with climatic parameters (precipitation and temperature), soil moisture content; factors that directly or

indirectly influence host-parasite relationship and propagation of environmentally transmitted helminths.

1.3. Ticks commonly associated with the African elephant

The African elephant, like other wildlife species, is infested by various species of ticks that are known to be competent vectors of many blood-borne pathogens. *Amblyomma thollonii* and *Rhipicephalus humeralis* are two species of ticks mainly found as adult stages in the African elephant and other pachyderms, including the rhinoceros, and hippos (Sikes, 1971; Dipeolu, 1976; Norval *et al.*, 1980; Horak *et al.*, 2010). These ticks are found in elephants in Kenya and other elephant ranges in Africa (Hoogstral, 1956; Dipeolu, 1976; Norval, 1983; Uilenberg *et al.*, 2013; Horak *et al.*, 2010). The two species of ticks are three-host ticks; immature stages are less host-specific and parasitize on domestic ruminants such as cattle, sheep and goats (Mackenzie & Norval, 1980; Gomes, 1993). Whereas the adults parasitize predominantly elephants and sometimes also found on rhinoceros which are considered an alternative host (Walker *et al.*, 2005; Horak *et al.*, 2017) as well as the giant forest hog and warthog (Uilenberg *et al.*, 2013).

1.3.1. *Rhipicephalus humeralis*

Rondelli first described *Rhipicephalus humeralis* in 1926. The specific name *humeralis*, from the Latin *humerus* meaning 'the shoulder' or 'upper bone of the arm', refers to the light-colored areas on the scapulae of the male (Rondelli, 1926). It is a three-host tick (Walker, 1957) that is mid-sized and ornate (Walker *et al*., 2005). It is commonly recorded in cattle and camels, among wildlife, it has predilection for the African elephant and Black rhinoceros (Walker *et al*., 2005). *Rhipicephalus humeralis* prefers hot, low-lying areas with an annual rainfall of 800mm to less than 400mm (Walker, 1957;

Walker *et al*., 2005). It has been recorded from various parts of southern Somalia eastern Kenya and northern Tanzania (Walker, 1957; Walker *et al*., 2005).

As described by Walker (1957), the male's (Figure 1.4.) are oval with widest part over halfway back. Ornate, color pattern often not very striking. Eyes often edged by a few punctations dorsally, otherwise flush with conscutum. Cervical pits deep and convergent. No cervical, lateral, marginal, postero-median or postero-lateral grooves present. Festoons well-developed. Punctation pattern made up of discrete medium-sized punctations with numerous small interstitial punctations. Medium-sized punctations tend to be concentrated on the scapulae. The conscutum is predominantly very dark brown in color with a pattern of light smoky-brown enamelled patches anteriorly. A light patch always appears to be present on each scapula and often between the cervical pits. Mottled white enamelling is present on the dorsal surface of the legs, particularly on III and IV. Capitulum. Longer than broad, the proportions usually being 5:4 dark brown in color. Basis capituli; broader than long. Ventral surface; relatively long and narrow, being over twice as long as broad. External margin long and straight, joining the slightly convex posterior margin in a smooth curve. Internal margin sinuous, joining the posterior margin in a right angle. Accessory anal plates absent (Walker, 1957).

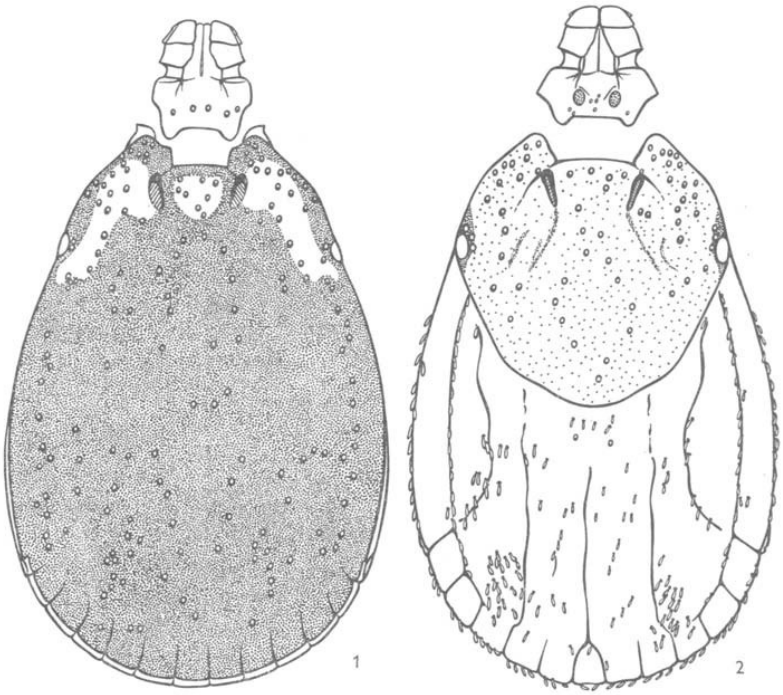


Figure 1.4. *Rhipicephalus humeralis* Rondelli 1926 Male: dorsal view of capitulum and conscutum (left). Female: dorsal view of capitulum, scutum and alloscutum (right) (©Walker, 1957)

The female description (Figure 1.4.) by Walker, (1957); Scutum; ornate and predominantly slightly brownish-cream in color with darker smoky-brown coloring round the eyes. Posterior margins slightly sinuous. Emargination wide and deep. Eyes with a few punctations along the dorsal edge. Long convergent cervical pits present but no true cervical or lateral grooves. Sometimes there are slight, rather smoky-coloured depressions in the position of the cervical grooves, as indicated by the stippling in (Figure 1.4.). Punctation pattern is made up of discrete medium-sized punctations interspersed with small punctations. Medium-sized punctations are grouped on the scapulae and often form rather ill-defined lines in the position of the lateral grooves (Figure 1.4.). Alloscutum; dark brown in color and bearing a number of white clavate hairs. Capitulum; brown

in color with slight enamelling on the basis capituli posterior to the palps. Basis capituli; twice as broad as long. Antero-lateral borders straight, posterolateral borders concave, lateral angles acute, approximately half-way back. Posterior margin straight and cornua strong. Porose areas oval, wide apart. The central part of the basis capituli enclosing the porose areas is usually sunken.

1.3.2. *Amblyomma tholloni*

According to Walker & Olwage, (1987), about twelve *Amblyomma* species have been shown to transmit *E. ruminantium*, another 5 species of *Amblyomma* ticks were shown to have abilities to transmit the disease albeit in the laboratory settings. Among these was *Amblyomma tholloni*, the elephant bont tick (Hoogstral, 1956; Walker & Olwage, 1987) whose adult is mainly found on the African elephant. MacKenzie & Norval, (1980) however indicated that the larvae and nymph stages of this tick were constantly found on cattle, sheep and goats within the Zambezi valley. Actually *A. tholloni* possibly transmits *E. ruminantium* to domestic stock (MacKenzie & Norval, 1980). Indeed, the distribution of *A. tholloni* is in tandem with the distribution of its main host, African elephant (Norval *et al.*, 1980; Hoogstral, 1956).

The morphological description of *A. tholloni* Neumann, 1899 male tick by Horak *et al* (2018), follows that, capitulum longer than wide. Festoons quadrangular, their dorsal surface practically confluent with that of the conscutum. Some specimens inornate, others with few to several patches of ivory-coloured to beige ornamentation surrounding an inornate central field. Eyes large, flat, pale brown, marginal. Cervical grooves deep, short, their inner margin concave; marginal grooves absent.

Numerous small, shallow punctations scattered along lateral conscutal margins, on dorsal surface of festoons and more sparsely on remainder of conscutal surface, which otherwise appears smooth. Legs brown, slender, faint ivory- coloured band surrounds distal end of each segment (Horak *et al*., 2018).

Further, Horak *et al* (2018) described the female *Amblyomma tholloni* tick's morphology (Figure 1.5.). An irregularly-shaped ivory-coloured patch of ornamentation with a tinge of iridescent green in each of the lateral fields and in the posterior field, some specimens may be inornate (Figure 1.5.) others with only a small patch of ornamentation close to the posterior tip of the scutum (Horak *et al*., 2018). Eyes large, flat, pale brown, marginal, slightly anterior to the widest part of the scutum. Numerous fine, long white setae on alloscutum. Distinct marginal groove commencing at posterolateral margin of scutum and delineating prominent festoons on alloscutum of unenorged females (Figure 1.9.). Legs less robust than those of male, segments brown with narrow, pale ivory-coloured band encircling distal end of each segment. Spiracular plates sub-triangular with convex angles, dorsal prolongation of perforated portion short, initially very broad, tapering slightly towards dorsal apex. Genital aperture V-shaped (Horak *et al*., 2018).

Figure 1.5. *Amblyomma tholloni* male (up) (from preserved specimen, Uganda strain) and *Amblyomma tholloni* female (down) (from preserved specimen, Uganda strain). Note that not all males and females of this species have such an extensive colour pattern as that shown here (©André Olwage).

1.3.2.1. Population genetics of *Amblyomma tholloni*

The genetic diversity, population genetic structure, and phylogeography of *A. tholloni* species is unknown (Mackenzie & Norval 1980; Petney *et al.*, 1987; Horak *et al.*, 2000; Horak *et al.*, 2003; Okino *et al.*, 2007; Uilenberg *et al.*, 2013; Horak *et al.*, 2017; Horak *et al.*, 2018). Fragmentation and isolation of the elephant populations across Africa could limit the gene flow and genetic diversity of this tick, on the other hand livestock movement could shape and reduce the genetic diversity among elephant populations. Several studies have focused on tick genetic diversity (de la Fuente *et al.*, 2005; Casati *et al.*, 2008; Ketchum *et al.*, 2009; Krakowetz *et al.*, 2010; Krakowetz *et al.*, 2011; Guglielmone *et al.*, 2013; Krakowetz *et al.*, 2014; Paulauskas *et al.*, 2016; Li *et al.*, 2017; Liu *et al.*, 2018), and tick population structure and potential factors influencing tick genetic variability (Lampo *et al.*, 1998; Mccoy *et al.*, 2003; Mixson *et al.*, 2006; Cutulle *et al.*, 2009; Beati *et al.*, 2012; De Meeus *et al.*, 2012; Dinnis *et al.*, 2014).

Factors predicted to structure genetic diversity in ticks are diverse and include: tick dispersal through host movement, habitat and climatic conditions that influence tick survival and distribution and the complexity of their life cycles, host specificity, and anthropogenic aspects that dictate selective pressures acting on vector populations (Hilburn & Sattle 1986; Barrett *et al.*, 2008; Kempf *et al.*, 2011; Maze-Guilmo *et al.*, 2016). Ticks that require two or three different host to complete their life cycle - are particularly interesting candidates for genetic population studies. First, the role that different host species play in their dispersal can vary depending on the host mobility and stage in the tick life cycle. Second, such ticks have the potential to transmit pathogens to multiple hosts and are potential conduits for novel pathogen spillovers and the emergence of human and livestock epidemics (Caron *et al.*, 2012; Eisen *et al.*, 2017).

While adult *Amblyomma tholloni* feeds predominantly on elephants, and sometimes on the hippopotamus, and rhinoceros and opportunistically on the giant forest hog and warthog

(Mackenzie & Norval 1980; Petney *et al*., 1987; Uilenberg *et al*., 2013; Horak *et al*., 2017), larval stages are less host-specific and parasitize domestic ruminants such as cattle, sheep, goats, and occasionally humans (Horak *et al.,* 2018). Nymph stages also parasitize wildlife species like African buffalo, lion, impala, and some species of birds (Horak *et al.,* 2000; Horak *et al.,* 2003; Horak *et al.,* 2018). A previous study suggests that the distribution of *A. tholloni* is limited primarily by the distribution of the African elephants (Horak *et al*., 2017). Since the African elephant populations are declining and are increasingly becoming fragmented, this pattern of host fragmentation is expected to lead to increased genetic isolation of their parasitic tick. The life cycle of a tick has an important influence on tick genetic diversity and population structure.

Tick species requiring two or three hosts spend most of their life cycle in the environment and for such species microclimate have an important effect in tick survival and gene flow between populations. In these ticks the extent of genetic differentiation will depend on host mobility and the degree of overlap between the home ranges of the host species. If host species are highly mobile but overlap little in their habitat-use, will be a greater proportion of the genetic variability of ticks to depend on host species and not habitat heterogeneity. On the contrary, if host mobility is limited, gene flow across habitats will be low. If gene flow is sufficiently low, selective pressures imposed by the habitat will be more important in determining the genetic structure of these ectoparasites.

For parasites with obligate multi-host life cycles, the selective forces experienced by different genotypes are likely to vary for different stages, given the requirement for establishing on potentially unrelated host species (Nadolny *et al*., 2015). Such trade-offs between fitness components might be expected to promote the maintenance of genetic polymorphisms owing to disruptive or fluctuating selection favoring different alleles in different obligatory hosts (Barrett *et al.,* 2008; Lampo *et al.,* 1998). Knowledge on patterns of genetic variation and

underlying evolutionary forces within populations of *A. tholloni* will be vital in creating a broader understanding of evolutionary factors and adaptation in ticks with complex life cycles dependent on multiple host species with varying degrees of host specificity in different ecological zones.

1.4. Haemoparasites infecting African elephants

Wildlife species harbor several important tick-borne hemoparasites such as *Theileria, Babesia, Ehrlichia* and *Anaplasma* where they may occur as asymptomatic infections. However, asymptomatic infections may progress to clinical disease when the host is exposed to ecological stressors such as co-infections with other pathogens (Munson *et al.*, 2008), translocation (Höfl*e et al*., 2004; Nijhof *et al.*, 2005), malnutrition (Wilson & Hirst, 1977) and drought. Clinical disease may increase overall morbidity and mortality, reduce fecundity and infant survival, factors that influence population performance and increases extinction risk for small endangered wildlife populations. For example, coinfection of Canine Distemper Virus with *Babesia* species in lions has been documented to cause lethal disease characterized by higher mortality in lions (Munson *et al.*, 2008). In rhinoceros and several species of antelopes, mortality associated with *Theileria* and *Babesia* infections has been shown to occur following capture and translocation (Nijho*f et a*l., 2003; Nijhof *et al.*, 2005). There are also cases where infections by *Theileria* in synergy with malnutrition have been implicated as a cause for high calf mortality and population decline in the roan and sable antelopes in South Africa (Wilson *et al*., 1974; Wilson & Hirst, 1977).

Previously, unknown species of *Babesia* were examined using microscopy in an African elephant in Kenya (Brocklesby & Campbell, 1963) and since then, no further publication by morphology or molecular techniques has been advanced.

1.4.1. *Ehrlichia* in elephants

The genera *Ehrlichia* and *Anaplasma* belong to the family Anaplasmataceae (Order Rickettsiales) and comprise of diverse species that infect and cause disease in a wide range of wild and domestic animals including humans and they are associated with an emerging group of zoonotic diseases. *Rickettsia* sp. Uilenburgi strain (Matsumoto *et al.*, 2007) have been detected in elephants while *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*), was linked to cowdriosis (Heart-water) disease in the African elephant (Walker & Olwage, 1987). Further, an experimental transmission of *Ehrlichia* spp. by *A. thollonii*, suggest that this elephant bont-tick is possibly among the species of *Amblyomma* ticks that transmit Heart water disease.

Many wild animal species have been identified as harboring *Ehrlichia ruminantium*, the rickettsial agent causing heartwater, a fatal disease of domestic ruminants in sub-Saharan Africa and eastern Caribbean (Perreau *et al*., 1980; Walker *&* *Olw*age, 1987; Peter *et al*., 2002; Vachiéry *et al*., 2008; Kasari *et al*., 2010). Heartwater causes high mortality in domestic ruminants (cattle, sheep and goats) in sub-Saharan Africa and in the eastern Caribbean (Walker & *Olwa*ge, 1987; Faburay *et al*., 2008; Vachiéry *et al*., 2008). An infectious but non-contagious tick-borne disease (i.e., heartwater) affects both domestic and wild ruminants. Heartwater infections often results into the death of clinically ill ruminants (Uilenberg, 1983; Bekker *et al*., 2001;).

Ehrlichia ruminantium is an aerobic, gram-negative, nonmotile, coccoid- to ellipsoidal-shaped organism in the order Rickettsiales and family Anaplasmataceae (Dumler *et al*., 2001). Mainly, the transmission is through infective blood (Camus *et al*., 1996). Upon entering the host's circulatory system, this obligate intracellular agent typically resides inside intracytoplasmic inclusions (diameter, ≤ 4.0 μm) in neutrophils and endothelial cells (Figure 1.6.) and replicates by binary fission and, less frequently, by budding. The number of replicated organisms

inside these cells (Figure 1.6.) can range from one to several thousand (Dumler *et al*., 2001; Prozesky *et al*., 1986).

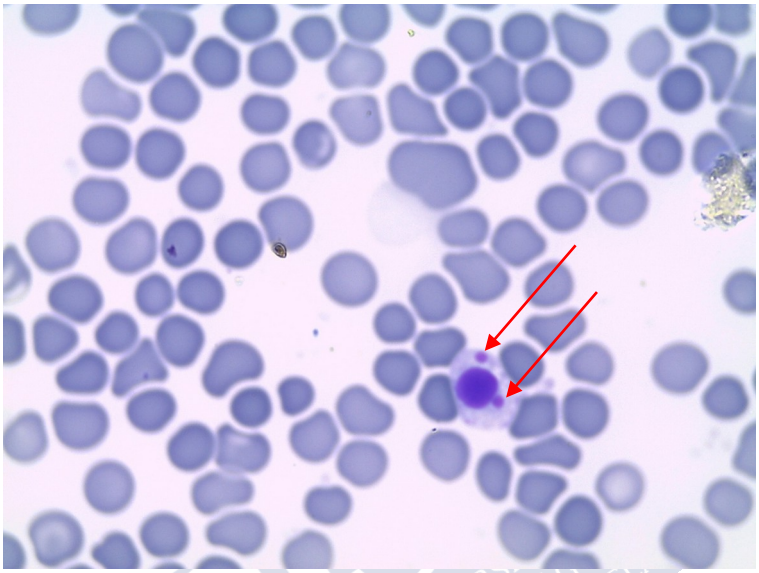


Figure 1.6. *Ehrlichia* on an elephant smear from Satao-Tsavo East National Park (Mg x 100)

In 2009, many sub-Saharan African countries and the island of Guadeloupe in the Caribbean reported clinical cases of heartwater in their domestic ruminant populations (Perreau *et al*., 1980; Neitz *et al*., 1986; Andrew & Norval, 1989; Wesonga *et al*.,2001; Vachiéry *et al*., 2008). Heartwater is caused by infection with the intracellular rickettsia *Ehrlichia ruminantium* (Dumler *et al*., 2001), formerly known as *Cowdria ruminantium*, which is transmitted by ticks of the genus *Amblyomma*, primarily *Amblyomma variegatum* and *Amblyomma hebraeum* (Walker & Olwage, 1987). *Ehrlichia ruminantium* also infects wild animal species, and several reports suggest a wide host-range spanning many taxa (Oberem & Bezuidenhout, 1987); for example; rodents (Theiler, 1962), wild angulates (Uilenberg, 1983), scrub hare (*Lepus saxatilis*) (Horak, 1986), and numerous ground birds that are hosts of *Amblyomma* tick larvae and nymphae (Theiler, 1962; Horak & Williams, 1986). The ability to have knowhow on

the dynamics of the spread of *E. ruminantium* and the precise host range is very vital in the; control of spread and to map out possible endemic areas (Oberem & Bezuidenhout, 1987). Such information is crucial in regulating international movements of animals (both wild, domestic) thereby controlling introductions of the disease vectors to new areas (Burridge *et al*., 2000). Indeed, Burridge *et al.* (2000) documented evidence of heartwater vectors in USA after an importation exercise of reptiles.

1.4.2. *Babesia* and *Theileria* in African elephants

Wildlife species harbor important tick borne disease such as *Babesia*, which mostly occur as asymptomatic. Babesias are place in the phylum Apicomplexa (Levine, 1971; Levine *et al*., 1980); class Aconoidasida (Mehlhorn *et al*., 1980); order Piroplasmodora (Wenyon, 1926); family Babesiidae (Poche, 1913); and genus *Babesia* (Starcovici, 1893).

Babesia, a tick transmitted parasite has very high economic importance and great veterinary and medical concern, as a matter of fact, *Babesia* is only second to trypanosomes, as the most common parasite found in mammalian blood (Starcovici, 1893; Schnittger *et al*., 2012). Babes discovered *Babesia* in the 19th century as a microorganism in erythrocytes of cattle in Rumania; he associated them with bovine hemoglobinuria or red water fever (Babes, 1888). In 1893 Smith and Kilborne, who also showed that *Babesia* was transmitted by a tick (Smith & Kilborne, 1893), gave the agent of Texas fever of cattle in the USA the name of *Pyrosoma bigeminum.* Actually, this was the first report of the transmission of a protozoan parasite by an arthropod. Still in 1893, Starcovici gave these parasites the names of *Babesia bovis*, *Babesia ovis* and *Babesia bigemina*, respectively (Starcovici, 1893; Mihalca *et al*., 2010). Koch, (1904) and Piana & Galli-Valerio, (1895) described other types of *Babesia* from the blood of other domestic animals, and were later to be known as *Babesia canis* and *Babesia caballi* from a

dog and a horse respectively (Piana & Galli-Valerio, 1895; Koch, 1904).

Currently, the knowledge on *Babesia* is vast due to advance in detection techniques such as microscopy, molecular techniques, and cell biology (Levine, 1988; Amici, 2001; Criado-Fornelio *et al*., 2004; de Waal & Van Heerden, 2004; Uilenberg, 2006; Lack *et al*., 2012). Indeed, Babesiosis in livestock causes a huge economic burden world over (Kuttler, 1988; Brown & Palmer, 1999; de Waal, 2000). In East Africa, bovine babesiosis or red water as it is commonly known is only second to East coast fever, another important tick borne disease in cattle caused by *Theileria parva* (de Waal, 2000). Vaccination and constant vector eradication by dipping cattle are effective control measures applied in the fight against the disease (Brown & Palmer, 1999; de Waal, 2000). In humans, babesiosis is an emerging tick borne disease is caused by *Babesia divergens* (cattle parasite) in Europe and *Babesia macroti* (small mammal parasite) in the United States (Wilson & Chowning, 1904; Healy & Ristic, 1988; Kjemtrup & Conrad, 2000). According to de Waal (2000), human babesiosis cases are often reported among human populations. In wildlife, *Babesia* was detected microscopically in a sick African elephant in Kenya (Brocklesby & Campbell, 1963). Moreover, endemic stability has been indicated in wildlife that have been infected by *Babesia* species (Penzhorn, 2006). Penzhorn, (2006) found that all lions within Kruger National Park were infected with *Babesia leo*, but they did not develop clinical disease.

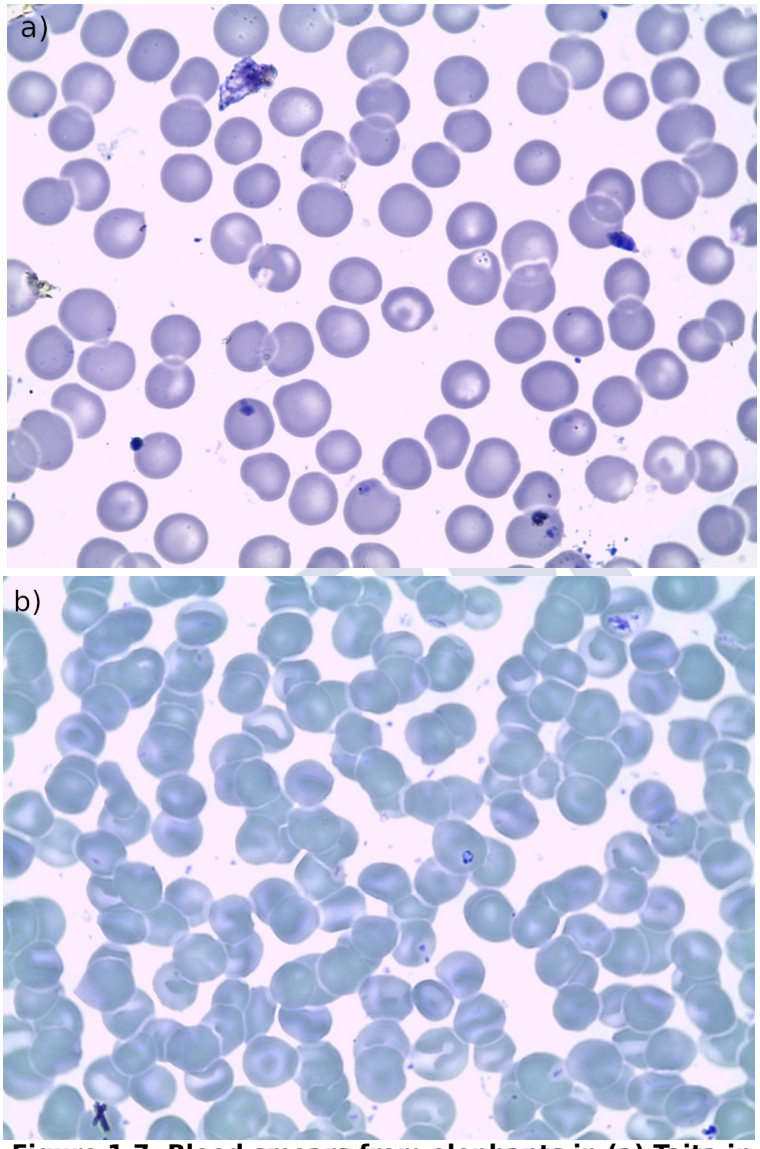


Figure 1.7. Blood smears from elephants in (a) Taita in Tsavo and (b) Solio in LSE showing *Babesia* (Mg x 100)

However, under stressful conditions, lions can develop clinical disease (Munson *et al*., 2008), equally so, Black rhinoceros that are known to have *Babesia bicornis* without developing clinical disease, can suffer from *Babesia bicornis* clinical disease after capture and confinement or translocation

(Nijhof *et al*., 2003; Nijhof *et al*., 2005; Penzhorn, 2006). In the US, small mammals and raccoons have been shown to have reservoir competence for *Babesia macroti* and therefore may be involved in disease transmission (Hersh *et al*., 2012).

Babesia and *Theileria* are commonly grouped together and referred to as piroplasma (Figure 1.7.), because the parasites after multiplication are often pear-shaped (Mehlhorn *et al*., 1984; Uilenberg, 2006).

Ixodid ticks transmit all species of *Babesia* (Smith & Kilborne, 1893). When a tick bites, sporozoites are injected into the host together with the saliva of the vector tick these sporozoites infect the erythrocytes (Figure 1. 7.).

Sporozoites develop into piroplasms. Multiplication usually results in two or four daughter cells, which leave the host cell and each enters another red cell (Figure 1.8.). Multiplication continues either until death of the host or until the immune system of the host stops the parasite replication (Mehlhorn & Schein, 1984). The most notable differences with *Theileria*, are the absence of extra-erythrocytic multiplication (schizogony) in *Babesia* and the cycle in the tick vector, which includes transovarial transmission in *Babesia* but only transstadial transmission in *Theileria* (Uilenberg, 2006).

Babesia parasites do not form pigment in the parasitized cell, which distinguishes them from other genera such as Plasmodium and Haemoproteus (Mehlhorn & Schein, 1984); apparently, *Babesia* species digest hemoglobin well so as not to leave any residues. In addition, the life cycle in both the vertebrate host and in the tick vector distinguishes them from other non-pigment forming protozoa, the most important ones being species of the genus *Theileria* (Rudzinska *et al*., 1984; Mehlhorn & Ahmed, 1994; Mehlhorn & Schein, 1998). *Babesia* parasites are defined (Figure 1.8.) and once injected in the host, enter directly into red blood cells (Mehlhorn & Schein, 1984).

In contrast, *Theileria* sporozoites do not infect red blood cells but penetrate a lymphocyte (or macrophage) in which they develop into schizonts (Mehlhorn & Schein, 1998; Mehlhorn &

Ahmed, 1994). The merozoites released from the schizonts enter red blood cells where they grow into non-pigment forming piroplasms and multiply by budding into four daughter cells, forming tetrads, often in the shape of a Maltese cross (Mehlhorn & Ahmed, 1994; Mehlhorn & Schein, 1998). Hence, by definition, *Babesia* only reproduces in the red cells, and this implies that as soon as schizogony is detected in the vertebrate host (Figure 1.8.) a given parasite can no longer be considered as a *Babesia* (Mehlhorn & Schein, 1984; Kakoma & Mehlhorn, 1994).

Both *Babesia* and *Theileria* piroplasms are infective to the tick. The tick becomes infected when ingesting blood cells containing piroplasms, which should probably be considered as gametocytes, as was already suspected in the early days (Wenyon, 1926). They develop into male and female gametes in the tick gut (Figure 1.8.). The microgametes fuse with macrogametes to form motile zygotes (Mehlhorn & Schein, 1984; Kakoma & Mehlhorn, 1994). In *Babesia* the zygotes multiply and the ''vermicules'' which result invade many organs of the tick, including the ovaries (Mehlhorn & Schein, 1984; Kakoma & Mehlhorn, 1994). Therefore, the infection passes through the ovary and the egg to the next tick generation (Kakoma & Mehlhorn, 1994) transovarial transmission (Figure 1.8.). The female tick always becomes infected and sporogony takes place in the salivary glands of larval, nymphal and/or adult ticks of the next generation (Mehlhorn & Schein, 1984). When the tick attaches to a new host, maturation of the sporozoites takes place and the host is infected with saliva from the tick. Certain species of *Babesia* can persist over several tick generations, even without new infections (Mehlhorn & Schein, 1984; Kakoma & Mehlhorn, 1994).

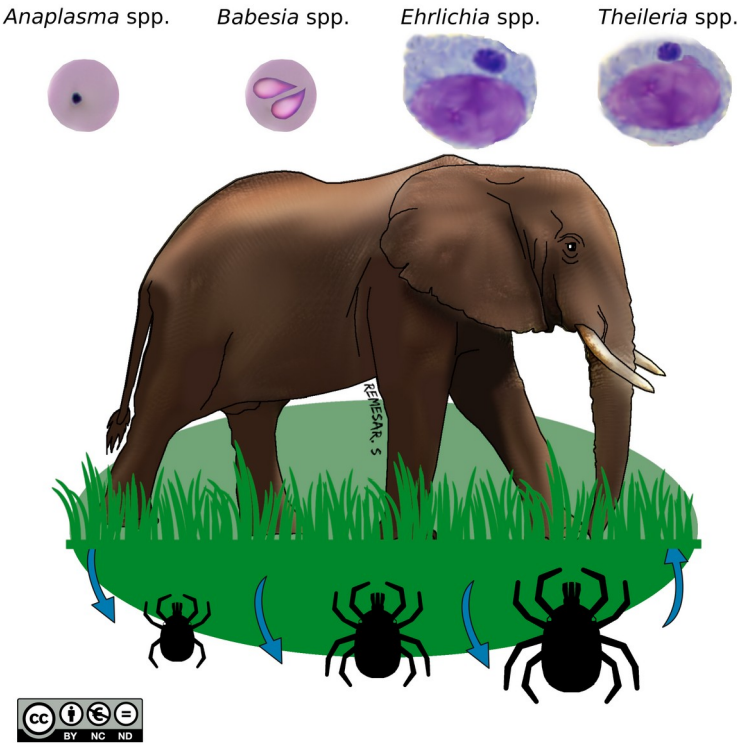


Figure 1.8. Generic life-cycle of *Anaplasma* spp., *Babesia* spp., *Ehrlichia* spp. and *Theileria* spp.

OBJECTIVES

2. GENERAL OBJECTIVES

Based on the exposed background, we set the following general objectives:

1st. To examine the relationships between helminths infection and within major elephant populations in Kenya spanning different agroecological zones.

2nd. To test the influence of age, social structure and Normalized Difference Vegetation Index, as the main drivers of these infection patterns.

3rd. Study the possible influence of elephant population on the genetic structure of *Amblyomma tholloni*; the elephant bont tick species in Kenya, using COX1 and the second internal transcribed spacers (ITS-2) of nuclear ribosomal DNA (rDNA) of *A. tholloni* as a genetic marker.

4th. Establish the patterns of geographic structure in *A. tholloni* due to host isolation and examine the inferences of historical tick demography and evolutionary forces driving genetic diversity.

5th. Determine the presence of haemoparasites and the prevalence of *Theileria, Babesia, Anaplasma* and *Ehrlichia* in elephants and ticks collected from the same hosts from different areas.

6th. Study the epidemiology of these pathogens in the African elephant identifying the role of the tick in the disease maintenance and spread.

MATERIAL AND METHODS

3. MATERIALS AND METHODS

3.1. Helminth infection in Kenyan elephant populations.

3.1.1. Study Area

The study was carried out in Tsavo East National Park (TENP), Laikipia-Samburu Ecosystem (TENP), Maasai Mara National Reserve (MMNR), and Amboseli National Park (ANP). The Figure 3.1. show locations of the four major elephant populations in Kenya.

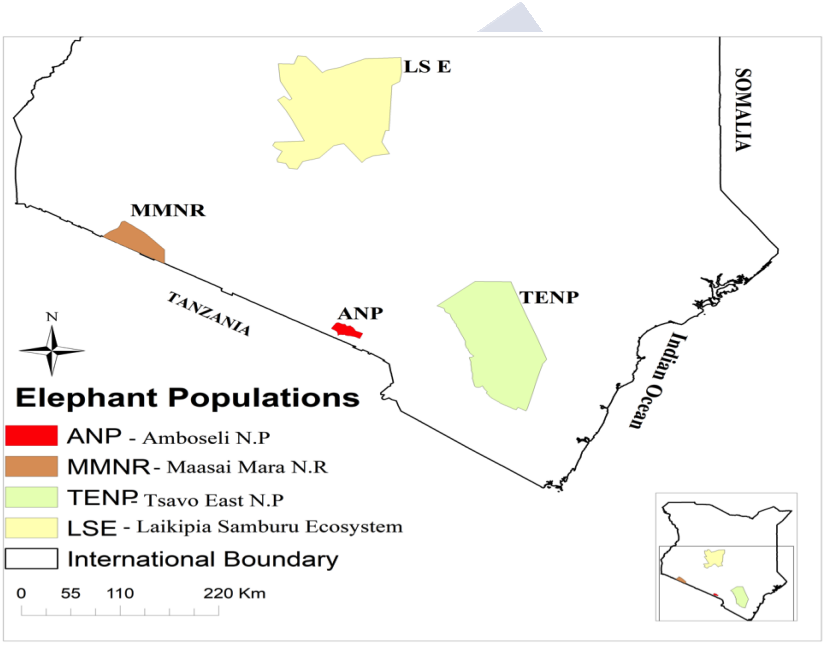


Figure 3.1. Map showing locations of the four major elephant populations in Kenya.

The four conservation areas hold the largest elephant populations in Kenya. These elephant populations are distinct with no connectivity.

Tsavo East National Park is situated in South Eastern part of Kenya. It is a semi-arid savannah that experiences bimodal annual rainfall pattern with long rains occurring in April-May while the short

rains occur in the November-December. Overall, rainfall is erratic and low with an average of 300-600 mm while temperature range is 20ºC-30°C. The area holds 7,727 elephants according to the 2018 census report by the Kenya Wildlife Service.

Laikipia-Samburu Ecosystem occurs on the central region of Kenya. The elephant census in 2017 carried out by Kenya Wildlife Service estimates 7,166 elephants. The annual rainfall ranges between 300 mm to 700 mm per annum. It is arid savannah grassland with bimodal rainfall, which mainly falls in April and November.

The Maasai Mara National Reserve is located on the southern part of Kenya along the border between Kenya and Tanzania, where it is contiguous with the Serengeti. Overall, the area is an expansive grassland savannah. The 2017 elephant census reported 2,493 elephants in this habitat.

Amboseli National Park is located at the base of Mount Kilimanjaro on the southern part of Kenya. This area holds 2,127 elephants according to the 2018 census report by the Kenya Wildlife Service. It is a generally an arid dry savannah open grassland land mixed with patches of scrubs and *Acacia xanthophloea* woodlands. Annual rainfall varies between 141–757 mm, with an average annual rainfall of 340 mm (data available at http://amboselibaboons.nd.edu/). A network of marshes resulting from underground recharge of the meltdown of Mount Kilimanjaro snowcap provides permanent water.

3.1.2. Sampling

Faecal sampling was carried out in the period of February-November 2017. Sampling was carried out through cross-sectional approach whereby dung samples from individuals in a social family herd, male bachelor herds or lone bulls were tracked until they defecated. The fresh dung bolus was carefully opened and approximately 20 g of the dung was scooped and preserved in 10% formalin.

The following information was collected for each sample; age of the individual or members of the social group (adult, sub-adult or juvenile), sex, date of collection, GPS coordinates at the time of

sighting, and type of social group whether female or male social group).

A female social group (Figure 3.2.) was defined as a group consisting of females and their offspring and occasional males whereas a male social group was defined as a solitary male (Figure 3.3.) or a group of two or males seen in proximity at the time of observation.

Figure 3.2. Female social group grazing in Tsavo East National Park

Figure 3.3. Solitary male elephant in Maasai Mara National Reserve

A total of 243 faecal samples; 71 from independent male groups or solitary males and 172 from family social groups were collected from the four study areas.

In terms of population, 62 family groups were sampled in Maasai Mara National Reserve (MMNR), 37 from Tsavo East National Park (TENP), 27 from Amboseli National Park (ANP) and 19 from Laikipia-Samburu Ecosystem (LSE). Additionally, male social groups in MMNR (n=19), TENP (n=22), ANP (n=16) and LSE (n=14) were sampled.

3.1.3. Parasitological Techniques

- Sedimentation

A method described by Vander Waal *et al.* (2014) was applied in principle but slightly modified in procedure. Approximately 4 grams of the elephant dung was weighed, mixed with 45 ml of tap water in a 50ml centrifuge tube and stirred until the mixture was slurry.

The dung slurry was sieved and left to stand for 30 minutes. Decanting and re-suspension of the sediment was repeated 2–3 times until the suspension was clear. A dropping pipette was used to place a drop (~0.05ml) of the sediment on glass slide for examination under a light microscope (Leica, DM500).

Presence of eggs of nematodes and trematodes were assessed and micrographs of at least 10 eggs of different morphotypes were taken.

The micrographs were processed using the imaging software for documentation and annotation (LAS EZ, Leica microsystems) to measure dimensions (length and breadth) of the parasite eggs.

- Flotation

Faecal samples were homogenized thoroughly with a stirring stick so that parasite eggs are uniformly distributed in the sample.

Initially, a faecal floatation fluid with specific gravity of 1.27 was prepared. Briefly, 454 g of table sugar was weighed and mixed with 355 ml of distilled water. The mixture was heated over low heat while being stirred until all the sugar dissolved. The slurry sugar solution was left to cool before use as the floatation fluid.

Faecal sample was homogenized as earlier described in sedimentation section and prepared by weighing approximately 4 grams of the elephant dung. The sample was mixed with 12 ml tap water, stirred and sieved through a tea-strainer and the filtrate transferred into a 15 ml plastic centrifuge tube.

When the filtrate was less than 15 ml, it was topped up with tap water and the tube capped followed by centrifugation at 1,500 rpm for 10 minutes. The supernatant was decanted out and the sediment re-suspended using flotation fluid added up to half of the tube. The sediment was mixed thoroughly with the flotation fluid using a stirring stick.

The tube was then filled to the top with more flotation fluid until it formed a slight bulging meniscus. A cover slip was gently placed on top of each tube ensuring the cover slip was centred on top of the tube. The tubes were centrifuged for 10 minutes at 1,500 rpm. After centrifugation, the cover slip was gently removed and placed directly on a clean glass slide for examination under light microscope (Leica, DM500).

Eggs of helminths were qualitatively assessed. Micrographs of at least 10 eggs of different morphotypes were taken and processed as described in the sedimentation section.

- McMaster

The helminth eggs were enumerated using a quantitative technique based on a calibrated McMaster chamber. The count is an estimation of the number of eggs per gram (epg) of the faecal sample. The faecal sample was prepared as described in the floatation section. A pipette was used to transfer the mixture to each of the two chambers of the McMaster slide. The preparation on the slide was left to settle for at least five minutes and then examined under a light microscope.

The eggs present on each chamber were counted and the total count for the slide multiplied by a constant figure of 50, to determine the number of eggs per gram.

3.1.4. Normalized Difference Vegetation Index (NDVI) Analysis

NDVI is a measure of vegetation reflectance and absorbance in the infra-red and blue spectra by green vegetation and is often used as an index of productivity as it is correlated with plant phenology, and nitrogen content. Overall, NDVI is strongly influenced by climatic parameters such as precipitation temperature and soil moisture. These factors directly or indirectly influence host-parasite relationship and transmission of environmentally transmitted helminths.

Data on NDVI were processed from satellite images obtained by a Landsat 8 satellite that uses the Operational Land Imager and Thermal Infrared Sensors for data capture. Satellite images of 30-meter resolution were retrieved from the Libra development seed website. Shape files of the study areas Amboseli, Maasai Mara, Laikipia-Samburu and Tsavo East, obtained from the Kenya Wildlife Service were used to clip the relevant satellite images.

Amboseli satellite images were downloaded from 168 path and 062 row in the period April-November 2017. Satellite images for Maasai Mara were downloaded from 169 path and 061 row in the period, December 2016 and January - March 2017.

Satellite images for the Laikipia-Samburu were downloaded from 168 path and 060 row in the period, April-August 2017. Satellite images for Tsavo East were downloaded from 167 path 062 row and from 163 path and 062 row for the period, October —December 2016; and January to March 2017. These periods coincided with the sampling months and three months prior to sampling.

The Tsavo East satellites images were mosaiced into a single image using the Q GIS software.

Selected satellite images were pre-processed using Q GIS to remove both radiometric and geometric errors. The corrected images were used to generate the Normalized Difference Vegetation Index (NDVI) at 250 X 250-meter resolution from 100 randomly selected points from each protected area. NDVI was calculated using this equation;

$$NDVI= (NIR –RED) / (NIR+RED)$$

Given that NDVI is a standardized method used to evaluate the health status of vegetation by quantifying the difference between near-infrared electromagnetic ray (which vegetation strongly reflects) and the visible red light (which vegetation absorbs), the result from the formula generates a value between -1 and +1, where negative values show water and values close to 0 show bare soils. NDVI values between 0.1 and 0.5 show low to medium vegetation density cover while 0.5 to +1 show high vegetation density.

To generate the NDVI raster, the calculator tool in Q GIS was used. Moreover, random points were generated within the study area, where the NDVI values for each random point were extracted. This was done because there was an assumption that elephant move within the habitat.

The generated NDVI values for the random point were compared with the sampled locations for the family and males as collected in the field.

3.2. Phylogeography and population genetic structure of the elephant tick, *Amblyomma tholloni* from Kenya

3.2.1. Study area

The study was carried out in four regions that sustain more than half of Kenya's savanna African elephant population. The areas included the Tsavo East National Park (TENP) in southern Kenya, the Maasai Mara ecosystem, the Amboseli ecosystem (Taylo*r et al*., 2019) in southern Kenya, and the Laikipia-Samburu Ecosystem (LSE) in north-central Kenya (Figure 3.4.). The TENP lies exclusively in the tropical warm semi-arid agro-ecological zone that receives annual rainfall from 400-600 mm with a 3-4 months' wet period. The main vegetation is scrubs, bushes, and grassland. The MME lies exclusively in the tropical cool sub-humid agro-ecological zone. The AMBE lies in a region with diverse agro-ecological zones whereby it's western

and parts of southern are within the tropical warm sub-humid agro-ecological zone that receives annual rainfall ranging from 600-1200 mm and experiences 4-6 months of the wet period.

The central, northern, and eastern parts of the MME lie within the tropical cool semi-arid zone that receives a relatively less annual rainfall of 400-600 mm and 3-4 months of rainfall a year. The main vegetation is constituted by scrubs, bushes, and grassland, except that the western and southern region where also woodland is present. The LSE has nearly 70% of its area in the tropical cool sub-humid agro-ecological zone which receives annual rainfall from 600-1200 mm and experiences 4-6 months of the wet period. Its vegetation is mainly bushland woodland, and grassland.

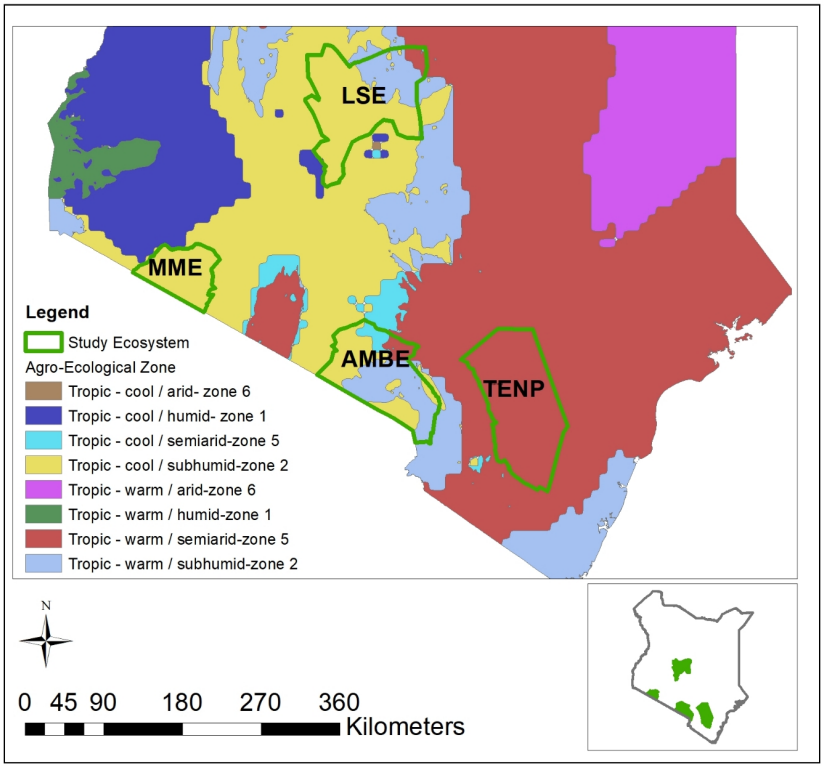


Figure 3.4. Map of Kenya (insert) showing the different agro-ecological zones that characterize the study areas with major elephant populations

3.2.2. Tick sampling and morphological identification

Adult ticks were collected from the ear and trunk of elephants immobilized for various clinical and population management interventions within the four study areas. The ticks from each elephant were placed in a labeled universal bottle and preserved in liquid nitrogen. Preserved ticks were transported to the laboratory where were thawed and identified under a stereomicroscope using morphological keys (Walker, & Olwage, 1987; Horak *et al*., 2018).

3.2.3. DNA extraction, PCR, and Sequence processing

A sub-sample of 137 ticks identified as *A. tholloni* were individually snap-frozen in liquid nitrogen followed by the retrieval and immediately ground to powder using pellet pestles (Sigma Aldrich, Missouri, United States) in 1.5 ml microcentrifuge tubes. The powder was then homogenized in 360 µl phosphate-buffered saline (PBS) (pH =7.4) and vortexed for 90 seconds. The extraction of total nucleic acids was done from 200 µl of the homogenate using DNeasy blood and tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions.

Extracted DNA was used in PCR amplification. To test for congruence of tick identities by morphological and molecular methods, we carried out a molecular analysis of a sample of 6 ticks by amplification of a 650 bp fragment of mitochondrial cox1 gene using primers LepF1 (5'- ATTCAACCAATCATAAAGATATTGG -3') and LepR1 (5'- TAAACTTCTGGATGTCCAAAAAATCA -3') (Hebert *et al*., 2004). For population genetic analysis, the 137 tick samples were analyzed through the study of a 1500 bp of the ITS2 gene-fragment using the primers F1-ITS2 (5′-CGAGACTTGGTGTGAATTGCA -3′) and (R1-ITS2 5′-TCCCATACACCACATTTCCCG -3′) (Chitimia *et al*., 2009).

Both PCR protocols were conducted in a total volume of 25 µl that consisted 1 µl of DNA template, 12.5 µl of OneTaq® Quick-

Load® 2X Master Mix with Standard Buffer (New England Biolabs-NEB, Massachusetts, USA) and 0.5 µl of 10 mM each forward and reverse primers. As a negative control, molecular grade nuclease-free water was used. The following cycling conditions were used for PCR amplification in a SimpliAmp thermal cycler (Life Technologies); an initial denaturation at 94 ºC for 1 min followed by 35 cycles of denaturation at 94 ºC for 20 s, annealing at 46 ºC for 20 s and 1 min extension at 68 ºC. The final extension was at 68 ºC for 5 min.

The PCR amplicons were resolved in a 1% (W/V) agarose gel by electrophoresis with a 1x TAE running buffer at 90 V for 35 min. The Gelpilot 100 bp plus ladder (Qiagen, Germany) was used as a molecular size DNA marker. The Ethidium Bromide stained gels were visualized under UV transillumination. All the amplicons with the expected band size were submitted for sequencing at Macrogen Europe B.V.

We visualized and edited DNA chromatograms and sequences using Geneious v11 (Kearse *et al*., 2012) software. Consensus tick-sequences for ITS2 and COX1 genes were generated from forward and reverse sequence data and exported as fasta files to MEGA X (Kumar *et al*., 2018) and DNAsp 6 (Rozas *et al*., 2017) for further alignment and analysis. Clean edited sequences were obtained for 98 ticks whereas the rest were discarded due to low quality chromatograms.

3.2.4. Molecular identification of *A. tholloni*

To test for congruence in identity between the morphological study and molecular analysis, similarity searches with data from the NCBI database using BLAST algorithm were performed. Thirty-six additional COX1 sequences from other species of ticks known to occur in Africa and combined them with sequences from GenBank that matched our sequences in the blast analysis were retrieved. A phylogenetic analysis using a maximum likelihood approach and 1000 bootstraps was calculated to determine branch support using MEGA X (Kumar *et al*., 2018) software. The General Time Reversible model with a gamma parameter (G = 0.861) and Invariables sites parameter

(I = 0.464) was the most parsimonious nucleotide substitution model based on 24 models tested using MEGA X software.

In addition, a phylogenetic analyses of orthologous sequences from different African ticks based on the ITS2 gene fragment from Genbank was performed by using the Maximum Likelihood method and Tamura 3-parameter model with a discrete Gamma distribution (5 categories (+G, parameter = 1.8557)). The tree was drawn from 34 nucleotide sequences with a total of 485 positions in the final dataset.

3.2.5. Genetic diversity, structure, and differentiation in *A. tholloni*

Gene polymorphism was estimated by computing gene diversity (Hd), number of haplotypes (h), nucleotide diversity (π), number of polymorphic sites, and the average number of pairwise nucleotide differences using Arlequin 3.5 (Excoffier & Lischer 2010). The determination of the haplotype number was performed using DNAsp 6 (Rozas *et al*., 2017). A Kimura 2-parameter model with a gamma parameter (G= 1.167) was the best model of DNA substitution selected from 24 models of base substitutions tested using the Akaike Information Criterion (AIC) in MEGA X (Kumar *et al*., 2018).

Evidence of population genetic structure was estimated using two pairwise estimates of population genetic structure, FST (Wright, 1949) and ΦST (Excoffier *et al*., 1992). The partitioning of global genetic variability within and between tick populations in Kenya based on FST (using haplotypes frequencies) and ΦST (using genetic distances) was conducted using Analysis of Molecular Variance (AMOVA) with 10,000 permutations in the ARLEQUIN software version 3.5 (Excoffier & Lischer 2010). For ΦST, pairwise distances were calculated using Kimura two-parameter model with the heterogeneity of the mutation rates, which are estimated to follow a gamma distribute on with shape parameter equal to 1.17 determined as the best nucleotide substitution model using MEGA X software. FST is based solely on haplotype frequencies, while ΦST takes into consideration both haplotype frequencies and genetic distances among those haplotypes. Thus, in cases where the evolutionary time is large

enough for these genetic differences to evolve, ΦST would enhance the ability to detect population structure. In contrast, when time is insufficient for haplotypes to diverge, inter-haplotypic distances will be small, even if the frequencies of these haplotypes differ, and only FST would detect population differentiation.

3.2.6. Natural selection and demography

We used DNASP 6.0 (Rozas *et al.*, 2017) to conduct numerous neutrality tests (Tajima's D, Fu's FS, Fu, and Li's D* and F *) on ITS2 sequence data from 98 ticks. Past population size changes were inferred by computing a raggedness index (r) obtained from a site mismatch analysis and by comparing the observed distribution to the distribution under the null model of constant population size. Tajima's D (Tajima, 1989) and Fu's FS (Fu, 1997) statistics are expected to be nearly zero in a constant-size population whereas significantly positive values indicate a recent population bottleneck, balancing selection or population subdivision. Negative and significant values suggest positive/purifying selection or genetic hitchhiking, and a recent spatial expansion or increase in population size (Fu, 1997). Fu and Li's D* and F * (Yun-Xin Fu & Li 1993) and R2 (Ramos-Onsins & Rozas 2002) statistics were also used to test for deviation of sequence variation from evolutionary neutrality.

Negative values of these statistics are most often attributed to positive selective sweeps, recent population growth, background selection, or genetic hitchhiking whereas positive values suggest population bottleneck or balancing selection. To confirm population expansion models, we employed raggedness index r (Harpending, 1994) and tested goodness of fit of the population expansion model.

The above tests exhibit varying levels of sensitivity in detecting potential causes, and used in combination, reveal evidence for or against specific evolutionary mechanisms.

TAJIMA'S D statistic, and Fu and Li D* and F* tests the most powerful in detecting a selective sweep and genetic hitchhiking (Simonsen *et al*., 1995). However, Fu's FS and R2 test are sensitive in detecting population growth but R2 is superior for small sample sizes, whereas FS is better for large sample sizes (Ramos-Onsins & Rozas 2002). Tajima's D and R2 are also superior when the intragenic recombination is considered (Ramı ´rez-Soriano *et al*., 2008). On the other hand, some popular statistics based on the mismatch distribution, and raggedness index r are very conserve.

3.3. Molecular identification of *Ehrlichia, Anaplasma, Babesia* and *Theileria* in elephants and their ticks.

3.3.1. Ethics statement

This study was permitted by the Research and Ethics Committee of the Kenya Wildlife Service (KWS/BRM/5001), the Institution mandated to protect and conserve Wildlife in Kenya. Blood samples were collected during scheduled interventions (clinical treatment and translocations) and involved experienced wildlife veterinarians who followed the approved protocols and guidelines on Wildlife Veterinary Practice 2006 and the Veterinary Surgeons Act Cap 366 of the Laws of Kenya that regulates veterinary practices in Kenya.

3.3.2. Study area

We conducted our study in four major ecosystems that sustain more than 50% of Kenya's elephant population. The locations include the Mara-Serengeti Ecosystem, the Amboseli Ecosystem (AMBE) and the Tsavo Ecosystem in southern Kenya and Laikipia-Samburu Ecosystem (LSE) in north central Kenya (Figure 3.5.).

The Mara-Serengeti Ecosystem (MSE), which lies between longitude 34˚ and 36˚ E and latitude 1˚, and 2˚ S and covers 25,000 km^2 consist of the Maasai Mara National Reserve and adjacent wildlife conservancies in Kenya and the Serengeti National Park and

associated game reserves in Tanzania. In this ecosystem, we focused on the Maasai Mara National Reserve and associated wildlife conservancies covering some 7000 Km^2, hereafter Maasai Mara Ecosystem (MME). Its annual rainfall ranges from 650 mm in the south east to 1,300 mm in the north west (Green *et al.*, 2015) and vegetation in the ecosystem is dominantly grassland with scanty cover of shrubs and thorny bushes (Walker & Olwage, 1987).

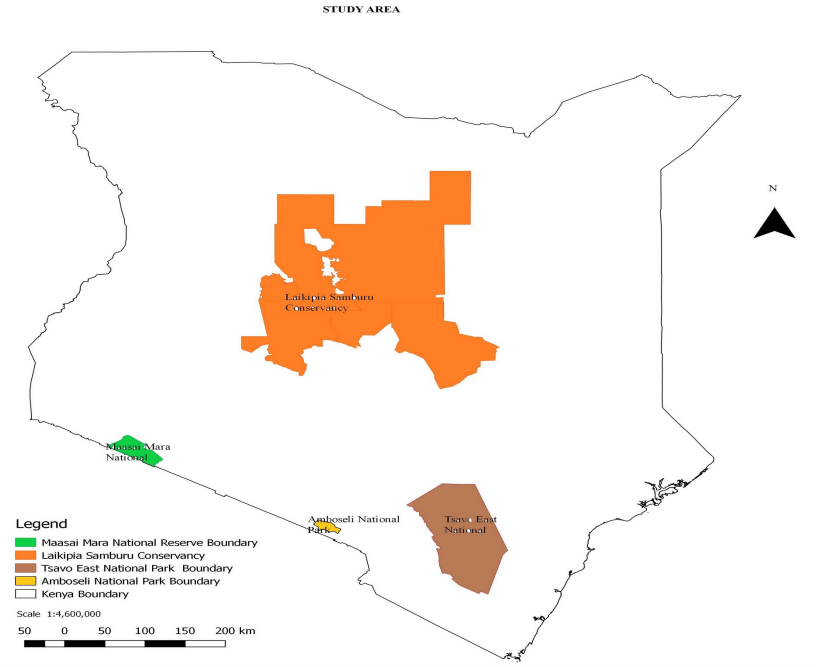


Figure 3.5. Map of Kenya showing locations of the sampled populations of the elephant and their tick.

The Amboseli ecosystem (AMBE) covers 8500 km2 and lies between longitude 36.75˚ and 38˚ E and latitudes 3˚ N and 3˚ S (Figure 3.9.) comprising Amboseli National Park, Chyulu National Park and adjacent group ranches and community wildlife conservancies. The ecosystem receives an average rainfall of 341-890 mm per annum and the area is characterized by swamps, wooded bushlands, open woodlands, and open grass- lands. Tsavo Ecosystem lies between 2–4˚S, and 37.5-39.5˚E and covers about 48,319 km2 including Tsavo East National Park, Tsavo West National Park, South

Kitui National Reserve and adjacent ranches and wildlife conservancies. Our sampling mainly focused on Tsavo East National Park (TENP), which covers an area approximately 13,000 km^2 and receives an annual rainfall that averages 300-600 mm per year and the vegetation is characterized by riverine formations, acacia grassland and mixed bushland (Agnew, 1968).

The Laikipia Samburu Ecosystem (LSE) lies between longitude 36-38.5 E and latitude 0-2.5 N and encompasses an area of 33,817 km2 consisting of Samburu, Buffalo Springs and Shaba National Reserves. LSE also includes several community and private wildlife conservancies including Ol Pejeta, Ol Jogi, Lewa, Solio Conservancies, Namunyak, Kalama, Meibae and Il Ngwesi. LSE receives an annual rainfall varying between 300-1250 mm per annum (Gadd, 2005). Vegetation cover is very diverse in this ecosystem and range from the lowland, xeric *Acacia* and *Commiphora* and scrub bush lands to the highland, mesic cedar and camphor forests.

These ecosystems are major strongholds of the Kenya's elephant population. Elephant total counts conducted between 2017 and 2018 show that the Maasai Mara and adjacent wildlife conservancies supports 2,493 elephants (Mwiu *et al.*, 2017), the Amboseli Ecosystem 2,127 elephants (Muteti *et al.*, 2018) and Tsavo East National park 7,727 elephants (Ngene *et al.*, 2017 a). The Tsavo ecosystem has 14,000, the largest elephant population in the country.

LSE has 7,166 elephants (Ngene *et al.*, 2017 b) the second largest population of elephants in Kenya after the Tsavo ecosystem houless CR (Thouless & Sakwa, 1995).

3.3.3. Sampling elephants for blood

The elephants were chemically immobilized for management interventions and other studies using Etorphine hydrochloride (Wildlife Pharmaceuticals (PTY) ltd) at a dosage of 14mg or 18mg for adult female and male respectively. A total of 104 blood samples were collected which consisted of 48 elephants from Tsavo East National Park, 28 elephants from the Laikipia Samburu Ecosystem, 20 elephants from the Maasai Mara National Reserve, and 8 from the

Amboseli National Park. Blood (~10 ml) was taken from the ear vein (Figure 3.6.) of the elephants was placed in an EDTA tube and 2ml aliquoted into cryovials and preserved in liquid Nitrogen while in the field and maintained frozen in a -40˚C Freezer until analysis.

Figure 3.6. Drawing a blood sample from the ear vein of an immobilized elephant in Laikipia-Samburu ecosystem

3.3.4. Tick sampling and identification

Engorged adult ticks were dislodged off from the ear and trunk of immobilized elephants. Ticks were collected from most individuals that were sampled for blood. The ticks from the same elephant were placed into a single cryovial. The ticks were preserved frozen in liquid nitrogen pending identification and analysis. Ticks were identified using morphological characteristics following published tick identification keys (Walker, 1957; Walker *et al.*, 2005; Uilenberg *et al.*, 2013). We focused mainly on the identification of *Amblyomma*

tholloni and *Rhipicephalus humeralis* because we were interested in examining the hemoparasites they carry being ticks commonly infesting elephants.

3.3.5. DNA extraction from elephant blood and ticks

DNA was extracted from 200 µl EDTA of elephant blood using DNeasy blood and tissue kit (QIAGEN, Hilden, Germany) as described by the manufacturer.

Ticks were individually processed. Each tick was placed in 1.5 ml tube and immersed under liquid nitrogen to freeze-dry and following ground into powder using pellet pestles (Sigma Aldrich, Missouri, USA). The powder was then homogenized in 360 µl PBS buffer (pH= 7.4) and vortexed for 90 seconds. Extraction of total nucleic acids was carried out from 200 µl of the homogenate using DNeasy blood and tissue kit (QIAGEN, Hil-den, Germany) following the manufacturer's instructions. Extracted DNA was then used in polymerase chain reaction (PCR).

3.3.6. *Theileria* and *Babesia* PCR amplification

Nested PCR targeting the 18S rRNA gene segment of *Theileria* and *Babesia* parasites from genomic DNA of elephant blood and ticks. Primary amplification of the target genes was carried out using ILO-9029 (5'CGGTAATTCCAGCTCCAATAGCGT-3') and ILO-9030 (5'-TTTCTCTCAAAGGTGCTGAAGGAGT-3') primer sets while the secondary amplifications were done using ILO-9029 (5'CGGTAATTCCAGCTCCAATAGCGT-3') and ILO-7782 (5'-AACTGACGACCTCCAATCTCTAGTC -3') primer sets as described by (Hawkins *et al.*, 2015).

The oligonucleotides used in this study were synthesized at Macrogen Inc., Europe. Primary PCR reactions were carried out in a 10 µl total volume that consisted of 5 µl HotStarTaq Master Mix (QIA- GEN, Hilden, Germany), 0.5 µl of 10 pmol/ul of each forward and reverse primers, 2 µl of the DNA template and 2 µl of sterile PCR water.

The cycling conditions included an initial denaturation at 95˚C for 15 minutes, followed by 30 cycles (95˚C for 30s, 55˚C for 30s, and an extension at 72˚C for 1 minute) and a final extension at 72 ˚C for 5 minutes in a T100 thermal cycler (Bio-Rad). The secondary PCR reaction volume was 25 µl consisting of 0.5 µl each of both primers, 1.0 µl of the primary PCR product, 10.5 µl of sterile PCR water and 12.5 µl of HotStarTaq Master Mix (QIAGEN, Hilden, Germany).

The cycling conditions used were identical to those of the primary PCR. The positive control was a *Theileria parva* positive sample obtained from a previous work in the laboratory while sterile PCR water was used as a negative control for the amplifications.

The amplicons were resolved alongside a Gelpilot 100bp plus ladder (QIAGEN, Hilden, Germany) in a 1.5% agarose gel stained with ethidium bromide and run 90 volts for 35 minutes before visualization under UV illumination. The size of the PCR product was about 450–500 bp.

3.3.7. *Ehrlichia*/*Anaplasma* PCR amplification

A nested PCR amplification targeting the Heat shock protein gene (groEL gene) of the Anaplasmataceae, was undertaken as described by (Park *et al*., 2005). GroEL is the highly conserved heat shock chaperonin protein used in phylogenetic relationship of bacteria and is better at differentiating *Ehrlichia* and *Anaplasma* species similar to the groESL gene consisting of 1,200 bp despite its short sequence 200-300 bp (Park *et al*., 2005). Primary amplification was carried out using EF1 (5'- CTG AYG GTA TGC AGT TTG -3' and ER2 (5'- AYR YYT TTA GCA GTA CC-3') primer sets while secondary amplification was done using EF3 (5'- GGT ATG CAG TTT GAY CG-3') and ER4 (5'- TCT TTT CTY CTR TCA CC-3') primer sets.

Primary amplification was done in a 10 µl total reaction volume that consisted 1 µl template DNA, 0.5 µl of 10pmol/ µl of each primer, 5 µl OneTaq1 Quick-Load1 2X Master Mix with Standard Buffer (New England Bio- labs-NEB, Massachusetts, USA), and 2 µl nuclease free PCR water. The primary amplification conditions included an initial denaturation at 94 for 1 minute followed by 20

cycles of denaturation at 94 for 20s, annealing at 50 for 20 sec and extension at 68 for 30s, followed by a final extension at 68 for 5 minutes in a T100 thermal cycler (Bio-Rad). The secondary PCR total reaction volume was 25 µl consisting of 0.5 µl each of both primers, 1.0 µl of the primary PCR product, 10.5 µl of sterile PCR water and 12.5 µl of HotStarTaq Master Mix (QIAGEN, Hilden, Germany). A positive *Anaplasma* spp. sample from other work in the lab was used as a positive control while sterile PCR water was used as a negative control for the amplifications. The conditions of the nested PCR were the same as those of the primary PCR. The PCR products, 300 bp in size, were visualized in a similar procedure as products of *Babesia*/*Theileria*.

3.3.8. Sequencing, editing, pathogen identification and phylogenetic analyses

All positive PCR products were purified and sequenced at Macrogen Inc., Europe in both the forward and reverse directions. Chromatograms for the forward and reverse sequences aligned and edited using SeqTrace and the poor-quality sequences were discarded (Stucky, 2012). The consensus nucleotide sequences were aligned using MUSCLE v. 3.8.31 (Edgar, 2004) algorithm in the MEGA X software. Unique sequences, herein referred to as haplotypes were identified from aligned sequences using DnaSP v 5.10.01 (Librado *et al.*, 2009).

Sequences with the highest similarity to our haplotypes were identified from GenBank (Benson *et al.*, 2009) using the BLASTn algorithm Altschul *et al.* (1990). In order to classify our haplotypes into species or clusters of species, at least two representative sequences of each known *Theileria, Babesia, Ehrlichia* and *Anaplasma* species from GenBank were obtained and combined to corresponding reference/matching sequences from GenBank for phylogenetic analyses. All edited *Theileria* and *Babesia* sequences from this study were deposited in Gen-Bank with accession number MN595045-MN595058 and all sequences for *Ehrlichia* were

deposited in GenBank with accession numbers, MN602332-MN602336.

All these sequences were aligned using MUSCLE v. 3.8.31 (Edgar, 2004) in MEGA X separately for *Theileria*, *Babesia*, and *Ehrlichia/Anaplasma*. The best model of sequence evolution and rate heterogeneity for the aligned genus specific sequences was estimated using MEGA X (Tamura et al., 2013). For *Babesia* spp. and *Theileria* spp. phylogenies, the best fit nucleotide substitution model was the Kimura 2-parameter model (Kimura, 1980) with a discrete Gamma distribution to model evolutionary rate differences among sites (*Babesia*; G = 0.469, *Theileria*, G = 0.204). The rate variation for some sites was allowed to be evolutionarily invariable in both *Babesia* (I = 34.6% sites) and *Theileria* (I = 47.1% of site) sequence evolution models. *Ehrlichia*'s best model for nucleotide evolution was Tamura 3-parameter model (Kimura, 1981) with a discrete Gamma distribution for evolutionary rate differences among sites (5 categories (+G, parameter = 0.209).

Phylogenetic relationships among *Babesia, Theileria* and *Ehrlichia* sequences were carried out using maximum likelihood analyses (MEGA X) and the evolutionary models and parameters stated above. The consensus tree topologies were evaluated for statistical support of internal tree branches using 1000 bootstrap iterations (Felsenstein, 1985). Nucleotide divergence or the average number of nucleotide substitutions per site between haplotypes from this study and sequences from known species from GenBank was estimated using the Jukes and Cantor model.

3.4. Statistical Analyses

Assigning eggs of strongyle nematodes into taxonomic classes using measurements is a challenge when there is some degree of overlap in the range of dimensions between taxa. Moreover, when the measurements are multidimensional, discordance in measurements taken in a single dimension can lead to bias in assignment. However, by using model-based clustering, these problems can be overcome as information on the variation in density of measurements across taxa

and the covariance of the different measurements is used to minimize assignment bias.

For our data we employed unsupervised multivariate cluster model using a Gaussian finite mixture analysis to group nematode and trematode eggs into operational taxonomic units (OTUs) using measurements of egg dimensions. The Gaussian finite mixture model (GMM), assumes that measurements of helminth eggs taken from each taxa (species or genera) will follow a normal distribution resulting in a (multivariate) Gaussian distribution with each taxonomic component forming a cluster with unique density, centred at the mean vector, and with other geometric features, such as volume, shape and orientation of measurements, determined by the covariance matrix. The volume, shape, and orientation of the covariance's can be constrained to be equal or variable across groups, giving rise to 14 possible models characterized by unique geometric characteristics (Scrucca *et al.*, 2016). The most parsimonious parameterisations of the covariance's matrices are obtained by Eigen-decomposition.

The Gaussian finite mixtures clustering process estimates a model for the data that allows for overlapping clusters, producing a probabilistic clustering that quantifies the uncertainty of observations belonging to components of the mixture. The unsupervised Gaussian finite mixture model was performed using the MCLUST package (Scrucca *et al.*, 2016) of the R statistical software (R-Core-Team, 2018).

The OTUs of nematode and trematode eggs produced were then assigned to taxonomic classes of helminth eggs based on mean length and breadth measurement from published records (Table 3.1.).

Table 3.1. Egg measurements (microns) of gastrointestinal nematodes and trematodes infecting African elephants compiled from the Literature

Parasites	Length (µm)	Width (µm)	References
Murshidia linstowi	[1]50	38	Van Der Westhuysen,1938
Murshidia longicaudata	[1]70	50	Van Der Westhuysen,1938

Murshidia hadia	[1]40	23	Van Der Westhuysen,1938
Murshidia africana	[2]60-[1]62	32-35	Van Der Westhuysen,1938; Fowler & Mikota, 2006
Murshidia omoensis	[1]60	30	Van Der Westhuysen,1938
Murshidia dawoodi	[1]65	35	Van Der Westhuysen,1938
Murshidia anisa	[1]48	30	Van Der Westhuysen,1938
Murshidia memphisia	[1]55-[3]73.25	35-40.82	Van Der Westhuysen,1938; Condy,1974
Murshidia loxodontae	[1]60	35	Van Der Westhuysen,1938
Murshidia asiza	[1]60	35	Van Der Westhuysen,1938
Murshidia soudanensis	[1]60	35	Van Der Westhuysen,1938
Murshidia brevicaudata	[1]55	32	Van Der Westhuysen,1938
Khalilia sameera	[4]39-78[8]	35-40	Monnig,1925; Ogden,1966
Quilonia khalila	[1]75	38	Van Der Westhuysen,1938
Quilonia loxodontae	[2]75-[1]82	38-44	Fowler & Mikota, 2006; Van Der Westhuysen,1938
Quilonia magna	[1]80	45	Van Der Westhuysen,1938
Quilonia apiensis	[1]83	48	Van Der Westhuysen,1938
Quilonia africana	[1]73	30-43[5]	Van Der Westhuysen,1938; Lane,1921
Quilonia uganda	[1]65	30	Van Der Westhuysen,1938

Quilonia brevicauda	[1]83	56	Van Der Westhuysen,1938
Quilonia ethiopica	[1]63	35	Van Der Westhuysen,1938
Mammomonogamus loxodontis	[6]100	40	Kinsella *et al*.,2004
Protofasciola robusta	[7]87.7-[3]94.1	48.5-61.52	Obanda *et al*.,2011; Condy, 1974
Brumptia bicaudata	[2]114	76	Fowler & Mikota, 2006

We present the data at the generic level rather than species due to variability in helminth egg sizes from different studies.

To test our hypotheses, we conducted both bivariate and multivariate analyses to test for variation in dependent covariates like prevalence and eggs per gram (epg) as well as independent covariates such as NDVI in the presence of unbalanced data. Any discordance in bivariate and multivariate models can indicate whether partial imbalance in data is causing spurious partial effects of covariates.

Using bivariate analyses, we tested for differences in epg across populations, with Friedman and Kruskal Wallis tests. We conducted multivariate analyses using Poisson and negative binomial GLM including hurdle and simple count models. The best model was selected based on parsimony using Akaike Information Criteria (AIC). To examine the influence of social group type, NDVI and age on epg we used the negative binomial hurdle Generalized Linear Model (GLM) with the glmmTMB package (Magnusson *et al.*, 2017) in the R Statistical software (R-Core-Team, 2018).

The statistical variation in prevalence of *Ehrlichia*, *Anaplasma*, *Babesia* and *Theileria* between elephant populations was evaluated using Chi-square tests performed using the R software for statistical computing (R-Core-Team, 2019).

RESULTS

4. RESULTS

4.1. Patterns of helminth infection in Kenyan elephant populations.

The best Gaussian finite mixture cluster model for trematodes was one with 2 components characterized by ellipsoidal, equal volume, shape and orientation (EE2) showing in Figure 4.1.

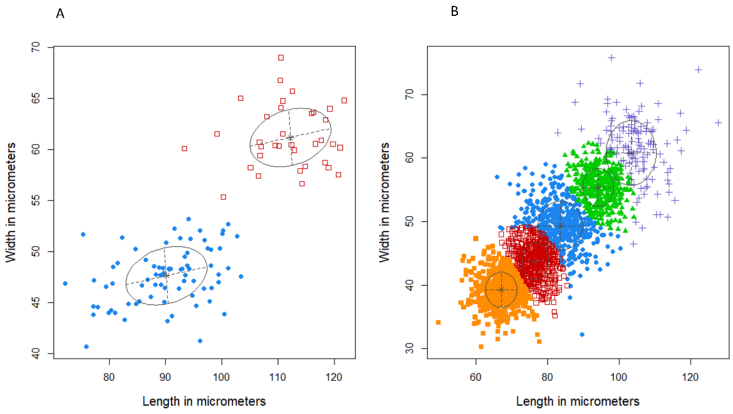


Figure 4.1. Model based classification of Elephant A) Trematode eggs and B) Stronglyle nematode eggs into operational taxonomic groups. Trematodes are classified into two operational taxonomic units (OTU1= blue, OTU2=red) and nematodes are grouped into five OTUS; OTU1=green, OTU2=Orange, OTU3=purple, OTU4=red and OTU5=Blue.

This model revealed that elephant populations in Kenya were infected with Trematodes consisting of two operational taxonomic Units (OTUs). Trematode OTU1 had egg length and width measurements like *Protofasciola robusta* and OTU2 had egg-measurements similar to *Brumptia bicaudata* based on published egg

dimensions of trematodes that infect African elephants (Table 4.1, Figure 4.2.).

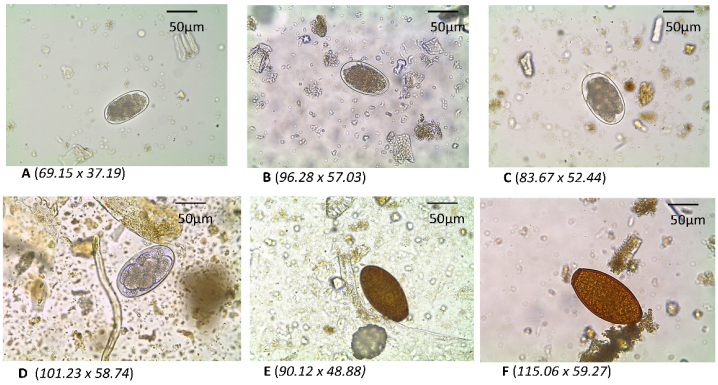


Figure 4.2. Nematode and trematode eggs representing the different sizes and genera (A: *Murshidia*, B & C represent the different sizes of *Quilonia* and D represent *Mammomonigamus* while E and F represent *Protofasciola* and *Brumptia*, respectively

Table 4.1. Results of unsupervised classification of trematode and nematode eggs into operational taxonomic units (OTUs)

OTUs	Mean ± Standard Deviation		Percentile (2.5 - 97.5%)		Range (Minimum - Maximum)	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)	Length (µm)	
Trematode OTUs						
OTU1	90.09 ± 7.47	47.61 ± 2.76	75.85 - 101.31	42.97 - 52.30	72.13 - 103.33	41-53
OTU2	112.06 ± 6.74	61.13 ± 3.11	98.42 - 121.07	56.50 - 67.08	93.37 - 121.73	55-69
Nematode OTUs						
OTU1	66.59 ±	39.06 ±	58.53 -	34.10 -	49.6 -	30-47

	4.01	2.64	73.67	44.20	77.7	
OTU2	83.25 ± 5.43	49.48 ± 3.39	71.86 - 93.79	42.81 - 56.87	65.9 - 100.4	32-59
OTU3	76.37 ± 3.35	43.51 ± 2.47	69.79 - 82.20	38.23 - 47.91	67.0 - 84.6	35-49
OTU4	94.36 ± 4.05	55.31 ± 2.87	87.00 - 102.50	49.70 - 60.80	84.9 - 104.9	48-62
OTU5	105±6	62±5	90-117	51-71	83-128	46-76

For nematodes, the best supported model based on BIC consisted of 5 diagonal components with equal shape (VEI) indicating five OTUs (Table 4.1.) one group putatively belonging to the genus *Murshidia* (OTU1) and three groups belonging to the genus *Quilonia* (OTU2, OTU3 & OTU4). On the other hand, OTU5 had large egg measurement outside the range for the genus *Quilonia* but similar to those recorded for *Mammomonogamus loxodontis* (Table 4.1.).

The prevalence of infection determined from sedimentation, was 97.5% whereas that obtained from floatation was 92.6% but the difference was not statistically significant ($\chi^2_{(1,\ n=243)}$ = 0.769, *P* = 0.366, Table 4.2.).

Table 4.2. Variation in prevalence of helminths across elephant populations and social groups in Kenya estimated using sedimentation and floatation methods

Elephant population	N	Floatation	Sedimentation
Male Social Group			
Amboseli	16	0.88	0.94
Laikipia Samburu	14	0.86	1.00
Maasai Mara	19	1.00	1.00
Tsavo East	22	0.86	0.91

Male group total	**71**	**0.90**	**0.96**
Family Social Group			
Amboseli	27	0.93	0.96
Laikipia-Samburu	46	0.98	1.00
Maasai Mara	62	1.00	1.00
Tsavo East	37	0.78	0.95
Family group total	**172**	**0.94**	**0.98**
Male and female Social groups combined			
Amboseli	43	0.91	0.95
Laikipia Samburu	60	0.95	1.00
Maasai Mara	81	1.00	1.00
Tsavo East	59	0.81	0.93
Grand Total	**243**	**0.93**	**0.975**

Therefore, all further analysis on prevalence were based on results obtained from sedimentation technique. The prevalence of helminth infection determined using sedimentation varied across different populations and this was statistically significant ($\chi^2_{(3,\ n=243)}$ = 8.972, P = 0.030) but there was no association of prevalence with elephant social groups (male social groups compared to female social groups, $\chi^2_{(1,\ n=243)}$ = 0.461, P = 0.497).

The prevalence of nematodes was 96.3% (95% CI= 93.09 – 98.29%) and it was significantly higher than that of trematodes which was 39.1% (95% CI=32.92 – 45.54%; $\chi^2_{(1,\ n=243)}$ = 179.18, P < 0.001). There was a lack of significant influence of social group ($\chi^2_{(1,\ n=243)}$ = 1.952, P = 0.162) and sampling location ($\chi^2_{(3,\ n=243)}$ = 5.956, P = 0.114) on prevalence of nematodes (Table 4.3.). In contrast, prevalence of trematodes was significantly influenced by the location or elephant

population ($\chi^2_{(3,\ n=243)}$ = 53.13, P < 0.001, Table 4.3.) but not social group ($\chi^2_{(1,\ n=243)}$ = 0.254, P = 0.614, Table 3.4.).

Table 4.3. Prevalence of nematodes and trematodes in male and family social groups across different populations estimated by faecal sedimentation method

Elephant population	N	Trematodes	Nematodes
Male social group			
Amboseli	16	0.44	0.94
Laikipia Samburu	14	0.79	0.93
Maasai Mara	19	0.42	1.00
Tsavo-East	22	0.18	0.86
Male social Group Total	71	0.423	0.930
Family Social Group			
Amboseli	27	0.41	0.96
Laikipia Samburu	46	0.76	1.00
Maasai Mara	62	0.19	0.98
Tsavo East	37	0.19	0.95
Family Social Group Total	172	0.378	0.980

The quantitative analysis by McMaster technique revealed that the mean number of eggs per gram of faeces (epg) was variable across elephant populations and between elephant social groups.

Bivariate analyses revealed that elephants sampled in family groups had significantly higher median epg than solitary males and/or males in bachelor groups controlling for epg variation across sampling locations or elephant populations (Friedman $\chi^2_{(1,\ n=243)}$ = 4, P = 0.046, Figure 4.3.). Elephant family groups from four elephant populations had significant differences in mean epg (Kruskal-Wallis $\chi^2_{(3,\ n=243)}$ = 40.942, P < 0.001, Figure 4.3.). Similarly, male groups from various elephant populations differed in mean epg (Kruskal-Wallis $\chi^2_{(3,\ n=243)}$ = 9.38, P = 0.025, Figure 4.3.).

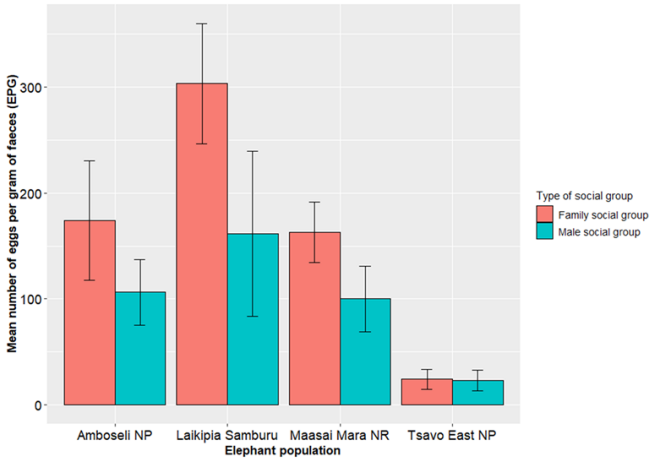


Figure 4.3. Mean of egg per gram of helminths for each social group in elephant populations in Kenya

Young males in mixed sex family groups had a lower epg than that of females (Table 4.4.) controlling for population or age but the differences were not statistically significant (Population: Friedman $\chi^2_{(1, n=243)} = 1$, $P = 0.317$; Age: Friedman $\chi^2_{(1, n=243)} = 2$, $P = 0.157$).

Table 4.4. Mean helminth epg for each sex and type of social group in Kenyan elephant populations

Sex and Social group	N	Mean	SD
Amboseli elephants			
Females in a family social group	19	202.63	318.62
Males in a family social group	7	121.43	236.04
Males in a male social group	16	106.25	125.00
Laikipia-Samburu elephants			
Females in a family social group	35	320.00	418.89
Males in a family social group	4	275.00	332.92
Males in a male social group	14	171.43	272.25
Maasai Mara elephants			
Females in a family social	46	145.65	204.62

group			
Males in a family social group	8	200.00	276.46
Males in a male social group	19	89.47	132.89
Tsavo East elephants			
Females in a family social group	25	36.00	66.96
Males in a family social group	10	0.00	0.00
Males in a male social group	22	22.73	45.58

Normalized Difference Vegetation Index (NDVI) was generally very low in areas occupied by study populations. The average NDVI over a 3-month period varied across the four study locations and these differences were statistically significant (Kruskal-Wallis; $\chi^2_{(3,\ n=243)} = 9.18$, $P = 0.027$). The lowest 3-month mean NDVI recorded was in Amboseli (mean + SD=0.091 + 0.002), and Tsavo East (Mean+ SD =0.118 + 0.006) and were relatively higher for Laikipia-Samburu (mean + SD=0.16 + 0.013) and Maasai Mara (0.239).

Table 4.5. A multivariate hurdle GLM model showing important factors explaining variation in epg among Kenyan elephant populations

Covariate	Estimate	Standard Error	z value
Count model coefficients (truncated negbin with log link)			
Intercept	3.09	0.92	3.35
3-month mean NDVI	24.18	10.04	2.41
Sub-adults & Juveniles cf. Adults	0.11	0.18	0.63
Family social group cf. male social group	0.29	0.18	1.68
Laikipia-Samburu cf. Amboseli	-1.43	0.72	-2.00
Maasai Mara cf. Amboseli	-3.74	1.50	-2.49

The most parsimonious multivariate model for variation in helminths epg was a hurdle glm model with a negative binomial distribution. This model indicated that the variation in non-zero positive counts of epg were driven by 3-months cumulative mean NDVI, social group type and elephant population or location of sampling (Table 4.5.). We observed a positive association between mean epg and a three-month cumulative mean NDVI (Figure 4.4.). Among elephant social groups, female social groups had a higher mean epg than male social groups (Figure 4.4.).

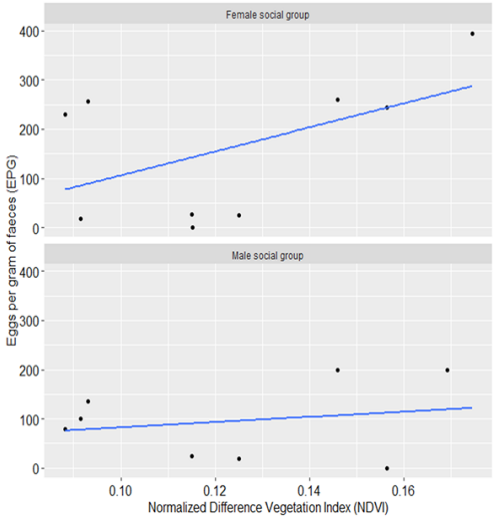


Figure 4.4. Scatterplot showing the relationship between NDVI and epg for each social group for all elephant populations combined.

Among elephant populations or protected areas, elephant population in the Samburu-Laikipia had a significantly higher epg than Amboseli while Tsavo elephants had a significantly lower epg than Amboseli (Figure 4.5.).

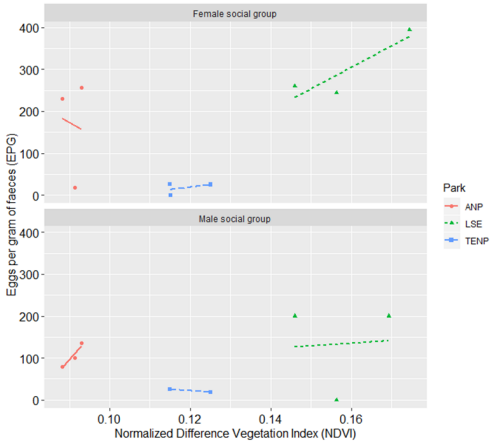


Figure 4.5. Scatterplot showing the relationship between NDVI and epg for each social group and elephant population treated separately.

However, Maasai Mara elephants were not different in epg from Amboseli. For the binomial part of the model which shows the presence or absence of a detectable epg, location and age and to a lesser extent NDVI had a significant influence on detectable helminth infection. Laikipia-Samburu elephants had a higher prevalence than Amboseli elephants whereas the elephants from Maasai Mara and Tsavo East had prevalence like that of Amboseli (Table 4.5.). Adult elephants had a higher detectable epg than sub-adults and juveniles combined.

4.2. Morphological identification of ticks and molecular confirmation of *A. tholloni*

A total of 698 mixed species of adult ticks were collected from the ear and trunk of elephants immobilized for various clinical and population management interventions within the four study areas.

Out of the 698 ticks, 97 ticks were from 9 elephants in AMBE; 162 ticks from 4 elephants in LSE; 100 ticks from 16 elephants in MME; and 242 ticks from 10 elephants in TENP. *A. tholloni* was the dominant tick (n= 268) of ticks infesting elephants representing 62% of ticks sampled in elephants. The second most abundant tick species was *Rhipicephalus praetextatus* which was dominant in TENP and represented 35% of all the sampled ticks (Table 4.6.).

Table 4.6. Percentage of species of ticks extracted from African savannah elephants in four locations in Kenya

	Amboseli Ecosystem (%)	Laikipia Samburu Ecosystem (%)	Maasai Mara Ecosystem (%)	Tsavo East NP (%)	Percent Total (%)
Amblyomma tholloni	89	53	100	26	62
Rhipicephalus praetextatus	10	47	0	67	35
Amblyomma gemma	1	0	0	0	0
Rhipicephalus humeralis	0	0	0	2	1
Rhipicephalus appendiculatus	0	0	0	5	2

A. tholloni ticks from our study and previous studies show variation in coloration patterns (Figure 4.6.). Specifically, noteworthy, is that some specimens of *A. tholloni* may be inornate, others with few to several patches of ivory-coloured to beige ornamentation surrounding an inornate central field.

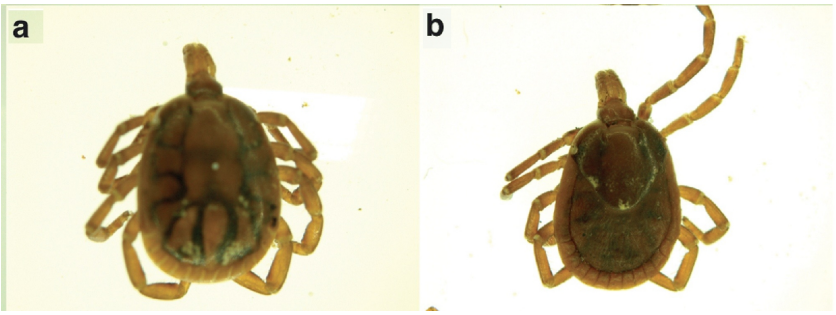


Figure 4.6. Photographed images of (a) *Amblyomma tholloni,* adult male; (b) *Am. tholloni*, adult female

From the 137 ticks for which DNA was extracted and the ITS2 amplified and sequenced, we obtained 98 clean sequences. In addition, out of the 137 ticks, a subset of 6 samples was sequenced at the COX1 gene (Figure 4.7.), but we recovered 5 clean sequences constituting 5 unique haplotypes.

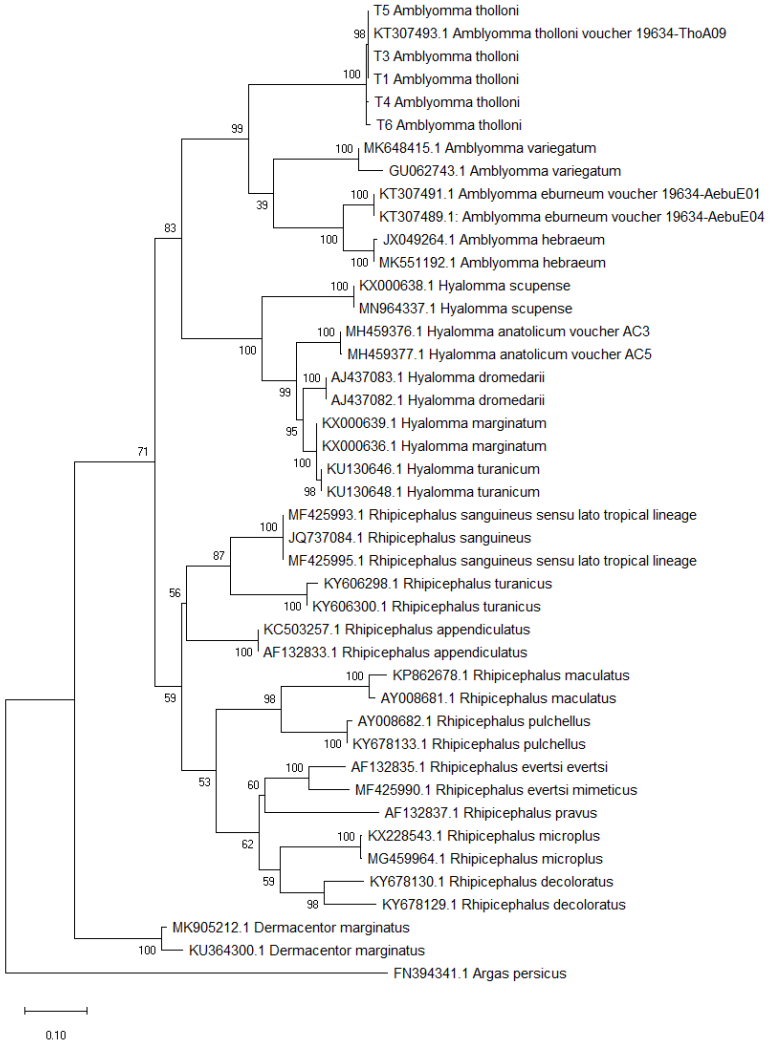


Figure 4.7. Phylogenetic relationships of different African ticks based on the Mitochondrial COX1 gene indicating the clustering of our tick samples with GenBank voucher samples for *A. tholloni*

Based on BLAST search on the NCBI database, our sequences of tick COX1 gene matched those of A. tholloni voucher specimens collected from Shimba hills, Kenya by 99-100 % identity (Table 4.7.). Further, a phylogenetic relationship involving African tick species with Argas persicus (soft tick; Family Argasidae) used as an outgroup confirmed the congruency of our morphological and molecular identification (Figure 4.8.).

Table 4.7. Haplotypes (T1-T6) of *Amblyomma tholloni* from the Kenyan elephants and their best matches to the Mitochondrial cox1 haplotypes in the GenBank

Haplotype ID	GenBank Accession number	GenBank species ID	E value	Maximum score
T1	KT307493.1	*Amblyomma tholloni*	0	1216
T3	KT307493.1	*Amblyomma tholloni*	0	1190
T4	KT307493.1	*Amblyomma tholloni*	0	1194
T5	KT307493.1	*Amblyomma tholloni*	0	1216
T6	KT307493.1	*Amblyomma tholloni*	0	1175

The ITS2 BLAST results showed that all tick sequences from this study had a 97-98 % sequence match to a questing *Amblyomma* sp. previously identified in the Maasai Mara National Reserve.-

In relation to genetic diversity and population structure of *A. tholloni*, the analysis of ITS2 sequences (Figure 4.8.) revealed 93 haplotypes based on 826 segregating sites (including insertions and deletions) observed in the 98 individuals sequenced.

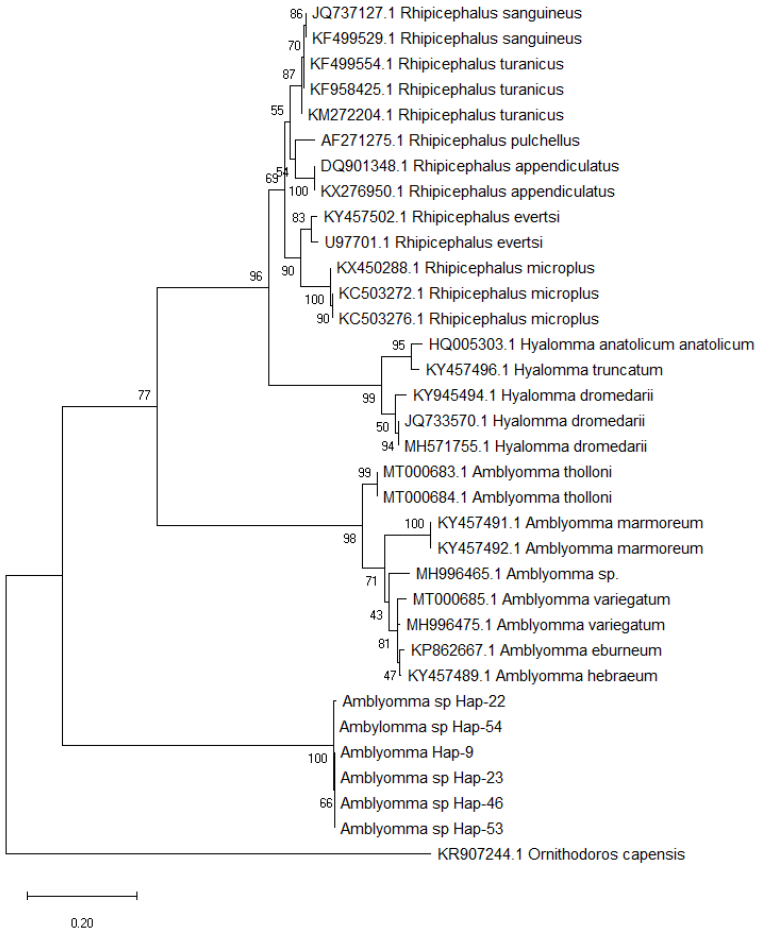


Figure 4.8. Phylogenetic relationships of different African ticks based on the ITS2 was inferred by using the Maximum Likelihood method and Tamura 3-parameter model with a discrete Gamma distribution (5 categories (+G, parameter = 1.8557)).

Of the 93 haplotypes, 89 were singletons, whereby one was shared among individuals within a geographical location and three were shared across different geographical locations (Table 4.8.). The global haplotype diversity (Hd) was 0.999 and all haplotypes in Amboseli, Laikipia-Samburu and Maasai Mara were different (Table 4.8).

Nucleotide diversity was moderate with a global average for all populations at 0.108 but nucleotide diversity of *A. tholloni* within each population ranged from 0.022 in Maasai Mara to 0.351 in the Amboseli ecosystem.

Table 4.8. Measures of genetic diversity indices (± Standard Deviation) within populations of ticks from different locations in Kenya as well as the overall value for all populations

	Amboseli ecosystem	Laikipia - Samburu ecosystem	Maasai Mara ecosystem	Tsavo East NP	Overall
Sample size	25	27	22	24	98
Number of Haplotypes, h	25	27	22	23	93
Number of polymorphic sites, S	707	523	128	417	826
Haplotype (gene) diversity, Hd	1.000 ± 0.011	1.000 ± 0.010	1.000 ± 0.014	0.996 ± 0.013	0.999 ±0.002
Mean number of pairwise differences	307.87 ± 136.19	135.07 ± 59.78	18.90 ± 8.70	85.66 ± 38.19	95.13 ± 41.23
Nucleotide diversity (average over loci)	0.351 ± 0.173	0.154 ± 0.076	0.022 ± 0.011	0.096 ± 0.048	0.108 ± 0.052

There was a weak but statistically significant genetic differentiation among the four tick populations (FST=0.059, P<0.001; ΦST =0.071, P<0.001), consistent with only 6% of the molecular

variance attributed to genetic variation between populations and the rest (94%) due to intra-population genetic variance (Table 4.9.).

Table 4.9. Partitioning of genetic variation within and between tick populations using haplotype frequencies (FST) or haplotypic genetic distance (ΦST)

Source of variation	Sum of squares	Variance components	Percentage of variation	*P*-value
F_{ST}				
Among Populations	535.818	4.4175	5.89	<0.0001
Within Populations	6633.818	70.57254	94.11	
Total	7169.636	74.99003	100	
Φ_{ST}				
Among populations	385.299	3.41231	7.05102	<0.0001
Within populations	4228.333	44.98227	92.94898	
Total	4613.633	48.39458	100	

In terms of pairwise genetic differences based on Kimura two parameter model, there was significant but low to modest genetic differentiation among populations. The fixation index, Φ_{ST} ranged from ~0.02 between Maasai Mara and Tsavo East National park to ~0.07 between Amboseli and Tsavo East National Parks (Table 4.10.).

Table 4.10. Pairwise Fixation indices (ΦST) between tick populations estimated using Kimura 2P

	Amboseli Ecosystem	Laikipia Samburu Ecosystem	Maasai Mara Ecosystem	Tsavo East NP
Amboseli		<0.0001	<0.0001	<0.0001

Ecosystem				
Laikipia Samburu Ecosystem	0.06982		<0.0001	<0.0001
Maasai Mara Ecosystem	0.04733	0.05971		0.009
Tsavo East NP	0.06066	0.05878	0.02127	

In respect to evolution and demography of *A. tholloni*, all populations of *A. tholloni* n Kenya had a significantly negative Tajima D and Fu & Li's F* and D* (Table 4.11.) suggesting positive selection, genetic hitchhiking, or a recent increase in population size. However, tests such Fu's F_S, Ramos-Onsins & Rozas's R_2 and raggedness index *r* which are sensitive to changes in demography were only significant for the Maasai Mara (Table 4.11.) indicating that this population experienced a demographic expansion.

Table 4.11. Values for various measures (p-value) of neutral evolution and measures of historical demographic change in ticks

Statistic	Amboseli Ecosystem	Maasai Mara Ecosystem	Laikipia-Samburu Ecosystem	Tsavo East National Park
Tajima's D, TD	-1.864 (0.003)	-2.173 (0.001)	-2.068 (0.004)	-2.586 (<0.001)
Fu and Li's D*, FLD*	-1.840 (0.025)	-3.358 (0.001)	-3.496 (0.005)	-4.165 (<0.001)
Fu and Li's F*, FLF*	-2.020 (0.011)	-3.239 (<0.001)	-3.391 (0.004)	-3.969 (<0.001)
Fu's Fs*[1]	-1.355 (0.145)	-9.879 (0.001)	-3.251 (0.043)	-3.184 (0.079)
Ramos-Onsins & Rozas's R2	0.103 (0.233)	0.100 (0.182)	0.090 (0.128)	0.159 (0.879)

Raggedness, r	0.007 (0.467)	0.009 (0.046)	0.006 (0.341)	0.008 (0.172)

*[*1]**The threshold for the significance of Fu's FS is 0.02***

4.3. Molecular identification of *Ehrlichia*, *Anaplasma*, *Babesia* and *Theileria* in African elephants and their ticks

Out of the 104 genomic DNA samples from Kenyan elephants amplified, only 13.5% (14/104) were positive for *Theileria* spp. or *Babesia* spp. based on gel electrophoresis.

The prevalence of these piroplasms appeared to vary among elephant populations, with a higher prevalence recorded for Tsavo East elephants and Maasai Mara elephants at 18.8% (9/48) and 15% (3/20) respectively. Piroplams were not detected in Amboseli elephants (0/8) but prevalence was 7% (2/28) for Laikipia-Samburu elephants.

This variation in piroplasm across populations was not statistically supported ($\chi 2$= 3.397, P = 0.334). Sixty-six elephant[1] samples were from males and 38 were from females but prevalence was higher among females (18%) compared to males (11%), although this was not statistically significant (χ^2 = 0.68, P = 0.409).

Six of the elephants (5.8%) were positive for *Anaplasma* or *Ehrlichia*. The prevalence of *Anaplasma* or *Ehrlichia* varied among elephant populations (χ^2 = 11.03, P= 0.012). A higher number of these were from Maasai Mara with a prevalence of 20% (4/20) followed by Amboseli with a prevalence of 12.5% (1/8) and TENP with a prevalence of 2% (1/48). There were no positive elephants in LSE (0/28).

A total of 52 ticks were captured. Fourty one were identified as *A. thollonii* (34 males and 7 females). Thirty-one of these ticks were from 20 elephants in the LSE, 5 ticks from 4 TENP elephants and 5 ticks from 3 AMBE elephants. The eleven remaining ticks were

identified as *R. humeralis* (10 males and 1 female) collected from 5 elephants in TENP.

No ticks were collected or identified from Maasai Mara National Reserve. Out of all the *A. thollonii* ticks, gel electrophoresis results indicated that 51% (21/41) were positive for *Theileria* spp. or *Babesia* spp. whereas 19.5% (8/41) were positive for *Anaplasma* spp. or *Ehrlichia* spp. Out of all the *R. humeralis* ticks, 27% (3/11) were positive for piroplasms (*Theileria* spp. or *Babesia* spp.), and 18% (2/11) were positive for rickettsia (*Anaplasma* spp. or *Ehrlichia* spp.).

There was no association between the positive results based on gel electrophoresis of PCR products for *Theileria* or *Babesia* (χ^2 = 19.07, P<0.0001). Similarly, there was no association between positive results in ticks and their elephant hosts for *Anaplasma* or *Ehrlichia* (χ^2 = 8.782, P = 0.003). In fact, there was only a case in which the tick and the host elephant were positive for one group of pathogens; piroplasm, but all the elephants sampled were negative for pirosplasm and one was positive for the *Ehrlichia* spp. Overall, the prevalence of piroplasms and *Erhlichia* spp. in ticks was higher than that of their elephant hosts. Sequence results and the GenBank BLAST search of 18S rRNA sequences revealed that elephants were infected with a single haplotype of *Babesia* (H1), which matched to a previously identified species of *Babesia* in elephants (Table 4.12.). No similar *Babesia* was identified in any of the ticks picked from elephants. However, the elephant bont-tick, *A. thollonii* had 7 haplotypes of *Theileria* spp. consisting of two different species; 6 haplotypes, H2-H7 matched *Theileria bicornis* and a single haplotype H8 matched *Theileria* cf. *vellifera*. The piroplasm sequences from *A. thollonii* had a 94-100% match to the closest GenBank sample (Table 4.12.).

There were no clean sequences of piroplasm recovered from *R. humeralis*. We did not detect *Anaplasma* in elephants or their tick ectoparasites. However, we detected five *Ehrlichia* haplotypes (H1-H5) from both the elephants and their ticks. All the five haplotypes identified closely matched to a single haplotype of *Ehrlichia* species previously identified from Japanese deer (Table 4.12.).

Table 4.12. GenBank Blast search results showing percent identity of the various piroplasm and rickettsia identified in ticks and their elephant hosts in Kenya. (E- value for each haplotype– 0.00).

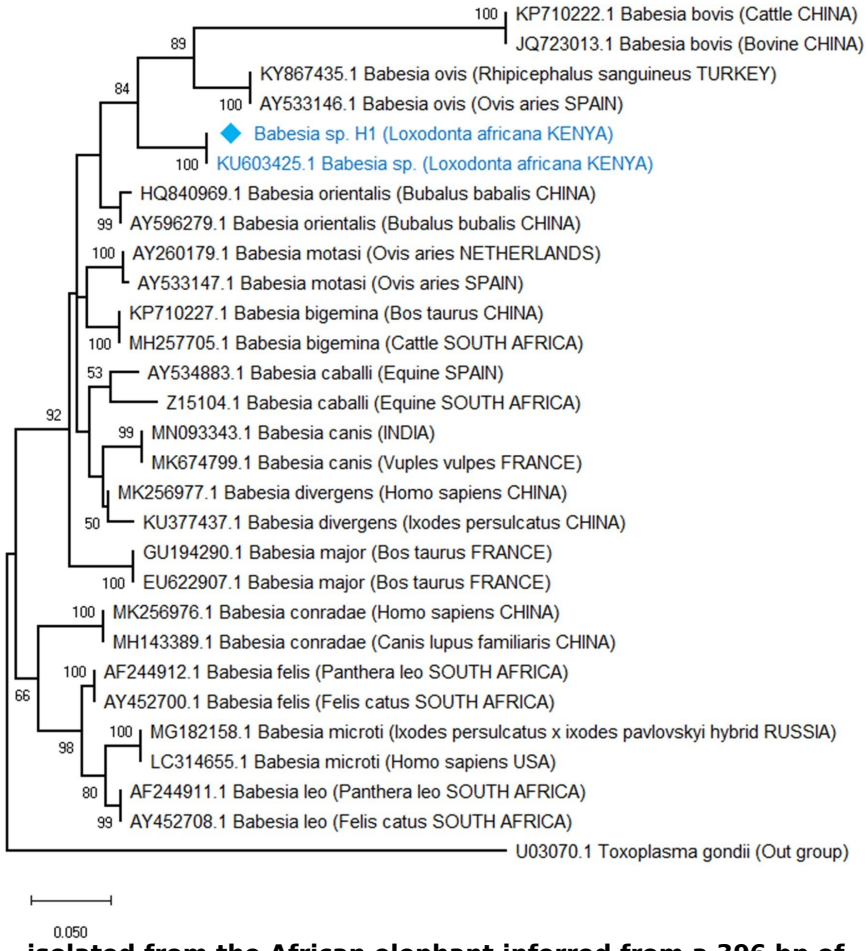
Haplotype ID	*Hosts*			Percent Identity	GenBank Accession Number
	Loxodonta africana	***Ambyomma thollonii***	***Rhipicephalus humeralis***		
***Babesia* spp. H1**	12	0	0	100%	KU603425.1
***Theileria bicornis* H2**	0	1	0	99%	MF536659.1
***Theileria bicornis* H3**	0	1	0	99%	MF536661.1
***Theileria bicornis* H4**	0	1	0	98%	MF536659.1
***Theileria bicornis* H5**	0	2	0	99%	MF536661.1
Theileria bicornis H6	0	1	0	100%	MF536659.1
***Theileria bicornis* H7**	0	1	0	99%	MF536661.1
***Theileria velifera* H8**	0	1	0	100%	GU733375.1
***Ehrlichia* spp. H1**	1	4	1	94%	AB454077.1
***Ehrlichia* spp. H2**	0	1	0	93%	AB454077.1
***Ehrlichia* spp. H3**	0	1	1	93%	AB454077.1
***Ehrlichia* spp. H4**	0	1	0	93%	AB454077.1
***Ehrlichia* spp. H5**	1	0	0	94%	AB454077.1

Percent similarity was, however, low (93-94% sequence identity match) with known GenBank sequences. *Ehrlichia* haplotype H1 was shared among the elephant, and its ticks; *A. thollonii* and *R. humeralis*. Haplotype H2 and H4 occurred only in *A. thollonii* whereas haplotype 3 was present in both tick species (Table 4.12.). Haplotype H5 was present only in the elephant (H5). Phylogenetic analyses revealed that the *Babesia* spp. detected in elephants is an unknown *Babesia* spp. previously detected in African elephants in Kenya with a 100% bootstrap support (Table 4.13. Figure 4.9.).

Table 4.13. GenBank accession number and percentage identities from this study

GenBank Accession Number	GenBank Species ID	Percent Identity
MN595058	*Ehrlichia* spp.	100%
MN602335	*Ehrlichia* spp.	100%
MN602334	*Ehrlichia* spp.	98%
MN602333	*Ehrlichia* spp.	98%
MN602336	*Ehrlichia* spp.	98%
MN595051	*Theileria bicornis*	99%
MN595050	*Theileria bicornis*	99%
MN595049	*Theileria bicornis*	99%
MN595048	*Theileria bicornis*	98%
MN595047	*Theileria bicornis*	99%
MN595046	*Theileria bicornis*	100%
MN595045	*Theileria velífera*	100%

Figure 4.9. Phylogenetic relationship of the *Babesia* species



isolated from the African elephant inferred from a 396 bp of the hypervariable V4 region of the 18S rRNA gene. The evolutionary relationships were inferred using the Maximum Likelihood method and the Kimura 2-parameter model with discrete Gamma distribution model for evolutionary rate differences among sites (5 categories (+G, parameter = 0.469) and a proportion of sites that are invariable (I = 0.346)

Theileria bicornis and *Theileria* cf. *vellifera* haplotypes also clustered with sequence matches from GenBank with a 95% and 100% bootstrap support respectively (Figure 4.10.).

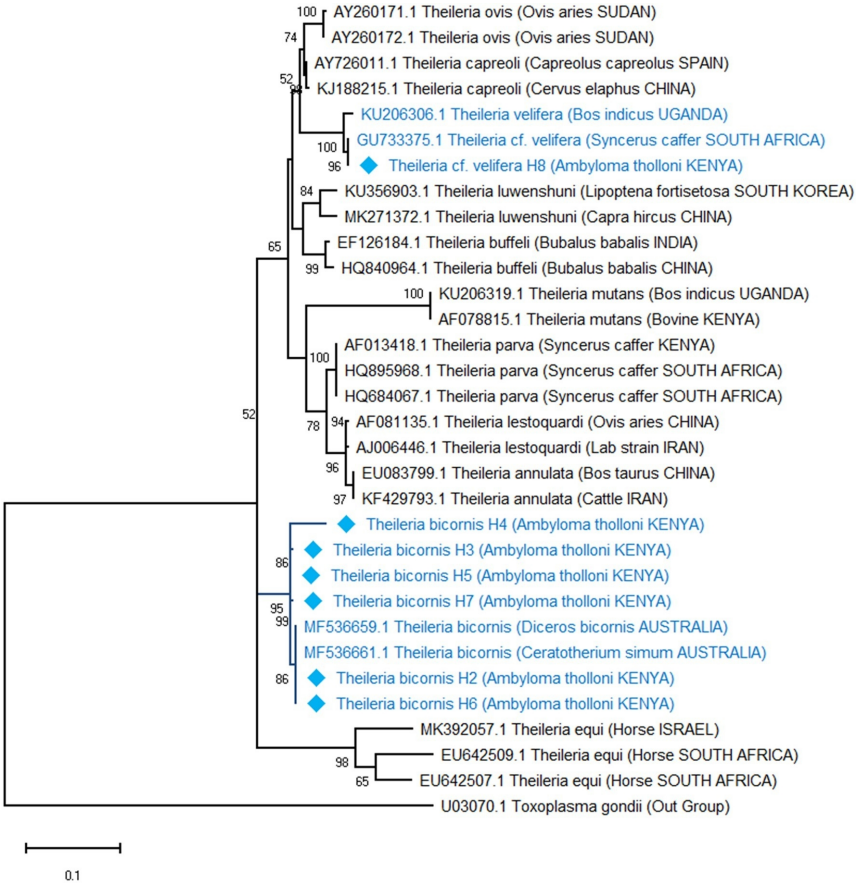


Figure 4.10. Phylogenetic relationship of *Theileria* species isolated from *Amblyomma thollonii* inferred from a 446 bp of the hypervariable V4 region of the 18S rRNA gene.

The evolutionary relationships were inferred using the Maximum Likelihood method and the Kimura 2-parameter model with discrete Gamma distribution model for evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.204) and a proportion of sites that are invariable (I = 0.471).

In contrast, *Ehrlichia* haplotypes did not cluster together with *Ehrlichia* spp. isolated from Japanese deer, which was its closest match from GenBank (Figure 4.11., Table 4.13.).

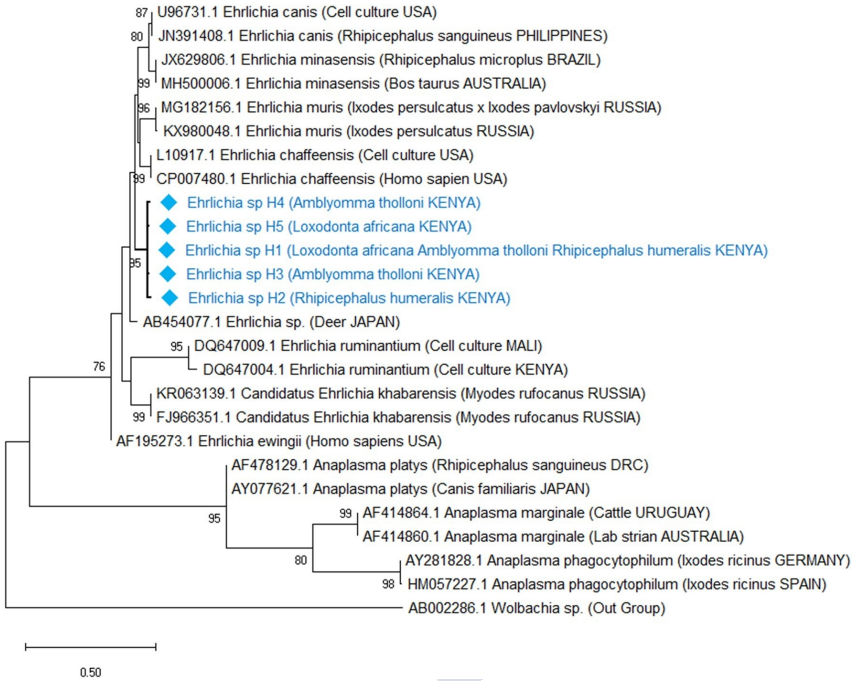


Figure 4.11. Phylogenetic relationship of *Ehrlichia* species inferred from 268 bp of the heat shock protein gene (groEL).

The evolutionary relationships were inferred using the Maximum Likelihood method and the Tamura-3 parameter model with discrete Gamma distribution model for evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.209).

However, all our haplotypes clustered together into a clade with 95% bootstrap support. Additionally, evolutionary distance analyses using the cantor model and assuming uniform rates of nucleotide evolution were consistent with both phylogenetic analyses and BLAST search matches (Table 4.13.).

DISCUSSION

5. DISCUSSION

5.1. Patterns of helminth infection in Kenyan elephant populations.

African elephant is a megaherbivore and a keystone conservation species whose ecological impact cannot be ignored. Its demography has continued to dwindle in many African range states where populations occur as separate sub-populations. As such, sub-populations may suffer different rates of parasite attack. Here we present the first study to examine helminth infection patterns across distinct African elephant populations and to evaluate factors associated with intra and inter population variability in prevalence and epg. This study is limited to the use of egg dimensions for helminth identification and inference of prevalence and load as obtaining worms is often invasive or opportunistic.

We identified two genera of trematodes, *Protofasciola* and *Brumptia* and two nematodes: *Murshidia* and *Quilonia* (tribe Quiloninea, Sub-family Cyathostominae). Using unsupervised classification, we recovered OTUs that had egg dimensions corresponding to species that are known to occur in East Africa, particularly Kenya. We specifically recovered eggs from species like *Protofasciola robusta*, *Brumptia bicaudata*, *Murshidia africana*, *Quilonia africana* and *Q. magna* and *Mammomonogamus loxodontis*, suggesting that with good information of egg measurements, it is possible to employ model-based clustering to group eggs into taxonomic units matching actual species.

The overall prevalence of helminths in elephants was 97.5%, however the pattern was characterized by a significantly high proportion of nematodes (96.3%) compared to trematodes (39.1%). In this study, we modified our sedimentation method that varied from most of the commonly used studies for examining flukes, where whole sediment is examined in a petri-dish under a dissecting microscope (Baines *et al.*, 2015). However, the obtained results are

comparable with infections in elephants elsewhere, which suggest the method did not significantly alter the sensitivity. For instance, in several studies, elephants have an infection pattern that usually involves 2-3 times higher nematode prevalence compared to trematodes. For instance, elephant populations in Burkina Faso, West Africa, had 97.7% mean prevalence of nematodes compared to 30.9% for trematodes (Nakandé *et al.*, 2007). A similar pattern was observed in Botswana, South Africa, where elephants had 100% prevalence of nematodes and 26% for trematodes (Baines *et al.*, 2015). This pattern seems not restricted to the African elephant, as comparable prevalence of nematodes (92%-96%) have been recorded in Asian elephants (Vidya & Sukumar, 2002).

Further, in our analysis, we observed a significant inter-population variation in prevalence, which was mostly driven by prevalence of trematodes. This is probably because trematodes, unlike nematodes, require the presence of an intermediate host, the snail, which depends on permanent water source with high calcium content. Specifically, elephants from the Laikipia-Samburu Ecosystem had a higher trematode prevalence while elephants of Tsavo East had the lowest prevalence. In contrast, Amboseli elephants that are known to be exposed to permanent water (Kioko *et al.*, 2006) had moderate levels of trematode prevalence than expected as compared to the Laikipia-Samburu population. Factors that determine trematode prevalence may be associated with variable environmental conditions that can influence abundance of snails, the intermediate hosts for trematodes (Lodge *et al.*, 1987). As expected, the prevalence of trematodes for Amboseli elephants that are exposed to permanent water, was nearly double that for elephants utilizing the seasonally water-logged Okavango delta where prevalence of trematodes was 23% (Baines *et al.*, 2015).

Mean epg was higher in family groups compared to male social groups, which is contrary to the male-biased parasitism, often linked to both hormonal and behavioural differences (Connors & Nickol, 1991; Zuk & McKean, 1996; Krasnov *et al.*, 2012; Guerra-Silveira & Abad-Franch, 2013; Martínez-Guijosa *et al.*, 2015). The hormone

testosterone is associated with immunosuppression in males, hence higher infection. According Thurber et al, (2011) there was no effect of testosterone on parasite burden when tested in male elephants in musth, individuals expected to have highest level of testosterone. It is likely that testosterone may not have an immunosuppressive effect in the elephant compared to other mammalian species (Baines *et al.*, 2015). In cattle, younger animals and males tend to have higher gastrointestinal parasite infection compared to older and female animals (Gunathilaka, 2018). Overall, in most mammals, males exhibit higher infection patterns compared to females as observed in humans, ungulates, rodents, bats and birds (Connors & Nickol, 1991; Zuk & McKean, 1996; Krasnov *et al.*, 2012; Guerra-Silveira & Abad-Franch, 2013; Martínez-Guijosa *et al.*, 2015). This suggests that elephant social structure has a significant influence on the mean epg because group living exposes its group members to higher infection risks compared to individuals who are solitary or form transient associations. This social dichotomy in infection pattern in the elephant may be related to habitat use and ranging patterns, which drive exposure and transmission of parasites such as helminths. In elephants, the ranging patterns of the female-led (matriarch) family groups are predictable often within reach of water since long-distance movement is avoided because of juveniles, whereas solitary males or bachelor groups have no such constraints and range for long distances (Barnes, 1982; Leggett, 2006; Shannon *et al.*, 2006; Mills *et al.*, 2018). Moreover, infectious helminth propagules build up in frequently used habitats, hence a higher risk of infection to family social groups (Hausfater & Meade, 1982). The influence of sociality on epg or egg shedding could also be attributed to gender composition in the group, especially the effect of male-bias to parasitism. However, we did not find significant difference in epg between male and female individuals in the family social groups.

Although the influence of social structure on epg has been observed in other elephant populations, epg in the current study was much lower. We found a mean epg of 172 in the female group and 89 in male group compared with elephant populations in the Okavango

Delta, Botswana where female groups had a mean epg of 1116 and males had 529 (Baines *et al.*, 2015). A study on male elephants in Etosha National Park in Namibia revealed that in one year, the average strongyle parasite egg counts (epg) varied between 1,409 and 2,204 in two different years (Coulson *et al.*, 2018). In addition, earlier studies on Rhodesian elephants recorded even much higher mean epg of 2072 with a range of 512-4,382 (Morgan & Van Dijk, 2011). Given that faecal egg count or epg is often used to assess parasite burdens, it has inherent pitfalls, being subject to numerous variables that confounds causation-effect relationship (Coulson, 2018). Few studies that have correlated epg to worm burden have shown variable outcomes (Seivwright *et al.*, 2004; Kim *et al.*, 2011; Cripps *et al.*, 2015; Gassó *et al.*, 2015). Therefore, we caution that the epg values we found in the elephant may not correspond to the total worm burden. A previous study apparently reports an average of about 30 000 worms per elephant and a range of 3,837-105,294 (Condy, 1974). Factors that influence the variations in the epg are not explicit but may include intrinsic parasite factors; variation in life histories of the infecting worm species, number of immature stages, worm gender imbalance as well as host - environmental factors (McKenna, 1981).

Our results show that NDVI; a measure of vegetation productivity, biomass and habitat structure, was variable but overall low across the four habitats. However, NDVI was positively correlated with the epg. Because NDVI is correlated with environmental variables like rainfall, soil moisture and habitat structure (Davenport & Nicholson, 1993; Nicholson & Farrar, 1994; Farrar *et al.*, 1994; Gamon *et al.*, 1995; Li *et al.*, 2004). It can directly or indirectly determine the distribution and abundance of infective stages of parasites, (i.e. rates of survival and transmission of infective stages, maturation of immature worms in the hosts and shedding rates of eggs), whether free-living or transported by intermediate hosts. Moreover, since NDVI is strongly correlated to primary production and nutritional content of forage (Tucker *et al.*, 1985; Prince, 1991; Paruelo *et al.*, 1997; Albayrak, 2008; Ryan *et al.*, 2012), it can influence the distribution and abundance of susceptible hosts, and can

enhance the heterogeneity of host spatial distributions (Sousa & Grosholz, 1991; Leimgruber *et al.*, 2001; Mueller *et al.*, 2008). In support, evidence of increased transmission of nematodes during the rainy season was shown in a study of African elephants Baines *et al.* (2015). Further a study on human helminth infection also found a positive relationship between prevalence of helminth infection with NDVI (Sousa & Grosholz, 1991).

5.2. Morphological identification of ticks and molecular confirmation of *A. tholloni*

We found four species of ticks attached to the ears and trunk of the Savannah elephants in Kenya including *A. tholloni* and *R. praetextatus*, the two dominant species constituting 97% of adult ticks recovered. The dominance of *A. tholloni* species was consistent with findings from a previous survey of ticks infesting African savannah (*L. africana*) and African forest elephants *(L. cyclotis)* (Kariuki *et al.*, 2019). Our morphological identity was congruent with molecular sequences for COX1 based on a voucher specimen collected from Simba Hills National Park in Kenya (Mwamuye *et al*., 2017).

In contrast, the closest matches to the ITS2 from this study (98%) was the undescribed *Amblyomma* sp. previously found in Maasai Mara (Oundo *et al*., 2020) and to another *Amblyomma* sp. from Lope National Park, Gabon (Lopez-Velez *et al*., 2015), and an *Amblyomma* sp. Extracted from the nostrils of a chimpanzees and a human in Kibale National Park, Uganda (Hamer *et al*., 2013). The best blast hit for the ITS2 gene fragment recovered from this study had an identity match of about 89-91% to *A. tholloni* species infesting African forest elephants from Gabon (Binetruy *et al*., 2020). The observed incongruence between the nuclear and mitochondrial genes have been associated with hybridization arthropods (Thielsch *et al*., 2017) as well as in different tick species such as *Ixodes*, and *Hyalomma* (Kovalev *et al*., 2016; Rees *et al*., 2003). Therefore, these discrepancies between mitochondrial and nuclear gene together with

the morphological variability in *A. tholloni* suggests that: the existence of a dichotomy of *A. tholloni* found in Savannah and forest elephants, the cryptic speciation in *A. tholloni* and that what appears to be morphologically *A. tholloni* is a species complex and ITS2 is not a good marker for the taxonomy of tick species particularly because of the possibility of recombination with less differentiated species complex.

The lack of resolution in the ITS2 gene fragment in resolving species in the genera *Amblyomma* and *Rhipicephalus* have been echoed by other researchers (Latrofa *et al.*, 2013; Mwamuye *et al.*, 2017). Findings from this study also suggest that the best match based on a blast search did not cluster together into one clade, confirming that the ITS2 fragment is not sensitive in the taxonomy of the genus *Amblyomma*.

The main objective of this study was to determine the genetic diversity and population genetic structure of *A. tholloni* and make inference of potential factors driving these patterns. This study revealed a high haplotype and nucleotide diversity among *A. tholloni* populations. However, the nucleotide diversity was more variable among populations. Ticks from Amboseli had the highest nucleotide diversity followed by Laikipia-Samburu Ecosystem. Tick populations in Tsavo East NP and Maasai Mara had the least nucleotide diversity. The pattern of nucleotide diversity appears to be related to the diversity of the agro-ecological zones in each location sampled. Ticks from the Amboseli ecosystem, which lies in a mosaic of five agro-ecological zones had nucleotide diversity of 0.3 while ticks from the Laikipia-Samburu, which occupies three agro-ecological zones, was 0.15.

Tsavo East NP and the Maasai Mara ecosystem, which are dominated by a single type of agro-ecological zones, had nucleotide diversity of 0.02 and 0.09, respectively. This pattern is consistent with the results of a study on *R. appendiculatus* across African countries in the great lakes region which revealed that 94% of the molecular variance in CO1 was explained by agro-ecological zones (Amzati *et al*., 2018). Tick species requiring two or three hosts spend most of

their life cycle in the environment off the host and for such species, microclimate will be important for tick survival and gene flow between populations. Therefore, ticks with different genetic makeup may be favored in different agro-ecological zones.

We found that the population of *A. tholloni* in Kenya had low levels of differentiation with only 6% of molecular variance accounted by geographical origin of the ticks and about 94% of the variation was within tick populations. In Kenya, the elephant populations sampled are all physically disconnected which has been confirmed by their genetic differentiation at the mitochondrial loci (Okello *et al.,* 2008). This result was unexpected and suggests that despite the isolation of elephant populations, there is connectivity among the tick populations. The exceptionally low levels of genetic differentiation amongst the tick populations suggest that the larval and nymphal stages of *A. tholloni,* which are less host-specific (Walker & Olwage 1987), may attach in livestock and get dispersed across regions. In Kenya, the pastoralist traverse regions with their livestock and share grazing areas with wildlife, which makes them potential agents for the spread of ticks. This means that the gene flow of this tick is depended on its young stages.

All the four populations of *A. tholloni* tick had an excess of rare haplotypes as indicated by the negative Tajima's D statistic suggesting recent population expansion, purifying background selection, or a selective sweep. However, tests of population expansion were weak for most of the populations suggesting that purifying selection is a major force shaping patterns of diversity among the *A. tholloni* populations. The larval stages of *A. tholloni* are less host-specific and parasitize on wild ruminants as well as domesticated species such as cattle, sheep, goats, and occasionally on humans (Horak *et al*., 2018) and therefore, likely subject to selection by acaricide use. Kenya has a long history of widespread acaricide use dating to the 1970s when their use was expanded countrywide (Crampton & Gichanga 1979).

Currently, acaricides are widely used in most of our study sites, which are rangelands with high-intensive commercial and community ranching farms that integrate both livestock and wildlife, or wildlife in

protected areas dispersing into community lands. Our results also bear resemblance in pattern to findings observed in South Africa, where authors noted that the majority of the rare haplotypes at the ITS2 of *Rhipicephalus microplus* were associated with multiple acaricide resistances (Robbertse *et al*., 2016). The impact of acaricides on tick populations is well known from a livestock production perspective globally (Lourens & Tatchell 1979; Kagaruki 1991; Norval *et al*., 1994; Keesing *et al*., 2013; Vudriko *et al*., 2016). However, its impact on the population genetics of many species of ticks is scarcely evaluated. Moreover, population genetic studies of ticks that demonstrate strong background selection on non-target genes may be misinterpreted as effects of recent demographic expansions (Robbertse *et al*., 2016; Amzati *et al*., 2018).

The routine use of pesticides for tick and mite control can lead to population declines, but when the population evolves resistance, these are followed by population expansions leading to outbreaks (Croft & Mcgroarty, 1973; Osakabe *et al*., 2009).

This study demonstrates morphological and molecular congruence in the identity of *A. tholloni* based on COX1 gene and unexpectedly inconclusive match using ITS. Further work is thus required to resolve the molecular taxonomy of this tick since it is a potential vector for important zoonotic pathogens as well as for critically endangered wildlife species (Mackenzie & Norval 1980; Matsumoto *et al*., 2007; King'ori *et al*., 2019).

This study revealed a high genetic diversity, across tick populations, which was correlated to the diversity of agro-ecological zones in each sampling locality. Despite the adults feeding mainly on elephants, whose populations in Kenya are spatially disconnected, gene flow was high across the four populations of ticks. Given that the young stages of the tick are less host-specific, they likely maintain connectivity across the populations, being passively carried by their hosts, sustaining the gene flow. Simultaneously, these immature stages could be subjected to selection pressure due to acaricide use on livestock. For these reasons, population viability and genetics of A.

tholloni might be mainly maintained by the immature stages, which are the agents of gene flow and selection.

5.3. Molecular identification of *Ehrlichia*, *Anaplasma*, *Babesia* and *Theileria* in African elephants and their ticks

Pathogen identification and knowledge of pathogen infection patterns in a vector-host system is vital for understanding disease dynamics and emergence. In this study, we investigated patterns of infection by *Theileria, Babesia, Ehlirchia* and *Anaplasma* in elephants and their ticks and identified these pathogens using molecular genetic tools. The prevalence of piroplasm in Kenyan elephants at 13.5% was lower compared with infection patterns in other large African wild hosts. For example, the prevalence of piroplasms has been recorded to be 100% in common zebra, *Equus quagga*, *Cape zebra*, *Equus zebra* and Grevy's zebra, *Equus grevyi* and in free ranging spotted hyenas *(Crocuta crocuta)* (Hawkins *et al*., 2015; *Burr*oughs et al., 2017; King'Ori *et al.*, 2018; Smith *et al.*, 2019) and between 92-100% in the African buffalo, *Syncerus caffer* (Oura *et al.*, 2005). However, the prevalence was comparable to that in South African black rhino, *Diceros bicornis* at 18.6% (Zimmermann, 2009). This was in contrasts to the prevalence of piroplasms in Black rhinos which had a prevalence of 43%, and white rhinos in Kenya with a prevalence of 66% (Otiende *et al.*, 2015). In Kruger National Park South Africa, white rhinos, *Ceratotherium simum* had a piroplasm prevalence at 36.4% (Govender *et al.*, 2011). The major driver for variation in prevalence across populations observed in the literature could be related to individual host susceptibility and the species of piroplasm involved. In all cases above the higher rate of prevalence involved *Theileria* but not *Babesia*, the species we mostly identified in our study. It is also apparent that the rate of prevalence is lower for rhinos

and elephants in the literature suggesting that elephants and rhinos are less susceptible to infection from tick borne pathogens compared to other species, and the ticks that are competent vectors for these diseases do not have elephants and rhinos as preferred hosts.

The prevalence of *Anaplasma* and *Ehrlichia* was much lower than for piroplasms at 5.8% but with significant spatial variation in prevalence. This is comparable to a prevalence of 6% for *Ehrlichia* in African buffaloes from Chobe National Park and the Okavango Delta in Botswana (Eygelaar *et al.*, 2015) and 5% for *Ehrlichia ruminatum* at Kruger National Park South Africa (Debeila, 2011). This contrast with a 0% rate detected in spotted hyenas and brown hyenas (Burroughs *et al.*, 2017).

Prevalence of piroplasms (*Theileria* spp. or *Babesia* spp.) in *A. thollonii* ticks was 51% but was 27% in *R. humeralis* ticks. The observed prevalence was higher than recorded for most locations. The prevalence of piroplasms in adult ticks of two species of the genus *Rhipicephalus* (*R. evertsi evertsi* and *R. decoloratus*), from the Western Oromia region in Ethiopia revealed an overall prevalence of 4% (8/202) *Theileria buffeli/sergenti/orientalis*, and 2% (4/202) *Theileria ovis* (Kumsa *et al.*, 2014). However, it was higher compared to a 2.7%; prevalence of *T. parva* infection in questing *R. appendiculatus* in the Tanga region, Tanzania (Swai *et al.*, 2006). The prevalence for *Anaplasma* spp. or *Ehrlichia* spp. was nearly identical in both *A. tholloni* and *R. humeralis* which was 19.5% and was 18% respectively. The prevalence was closely similar to a prevalence for *Anaplasma* and *Ehrlichia* infection rates of 16.4% from *Amblyomma* ticks parasitizing cattle and sheep in Ethiopia (Teshale *et al.*, 2015).

Overall, we observed that prevalence of *Erhlichia* and piroplasms was higher in tick vectors compared to the elephant. These findings contrasts those for the prevalence of *Theileria parva* between the tick, *Rhipicephalus appendiculatus* and their cattle host in Kenya where the infection rates for cattle compared to ticks was 43.5% and 2.3% in Limuru and 33% and 11.5% in Kakamega (Watt *et al.*, 1998). These differences may suggest strong immune selection of parasites by the elephant host than in the vector. This differential selection can

be expressed as variation in pathogen load or DNA concentration between the vertebrate host and arthropod vector. Alter- natively, these results may suggest that the reservoir vector for tick borne pathogens infecting elephants are tick or other species and elephants are a sink host.

Our results revealed infection of Kenyan elephants with a *Babesia* spp. that is phylogenetically different from most of the commonly known species available in public genetic databases. This species was however identical to a species recently identified from Kenyan elephants and deposited in GenBank (Accession numbers; KU603425 and KU603424) by some of our colleagues. There are no other recent records of piroplasms in the elephant except for the historical information, on the description and identification of *Babesia loxodontis* (Rodhain, 1936) and later by Brocklesby and Campbell (1963) from a sick elephant in Kenya using microscopy. The effect of Piroplasm infection on the health of the African elephant is not apparent but historical information suggests that it causes debilitation (Brocklesby & Campbell, 1963). Moreover, Fowler and Mikota (2006), suggests that *Babesia* spp. infection is prevalent in Asian elephants where they are associated with weakness, fever, jaundice, constipation and haemoglobinuria.

We did not detect *Babesia* species in the ticks detached from *Babesia* infected elephants but rather, the ticks were infected with *Theileria* spp. It was of interest that the ticks did not share the *Theileria* spp. with the host elephant. Specifically, we identified *T. bicornis* in adult *A. thollonii* ticks that infested the elephants, but this piroplasm was not detected in elephants that hosted these ticks as well as the overall elephant population examined. Moreover, *T. bicornis* is an important piroplasm known to cause fatal infection in the endangered black rhinoceroses (Nijhof *et al.*, 2003) but its tick vector is still unknown. Tick species that preferentially infest rhinoceros, *Dermacentor rhinocerinus* and *Amblyomma rhinocerotis* (Knapp *et al.*, 1997; Nijhof *et al.*, 2003) as well as *Rhipicephalus evertsi evertsi* (Mans *et al.*, 2015) have been suspected as potential vectors.

Our result is the first record of *T. bicornis* identified in a tick, and suggests *A. thollonii* as potential vector, though experiments on its vector competence are recommended. Further, absence of the parasite in elephants that hosted the infected ticks could imply that the parasite does not establish in the elephant. In addition, *A. thollonii* being a three-host tick, whose adult stage preferentially feeds on elephants, may have acquired the *T. bicornis* infection from rhinoceros in its earlier developmental stages (transtadial perpetuation) before infesting the elephant. *T. bicornis* is highly prevalent in the population of white and black rhinoceros in Kenya (Otiende *et al.*, 2015) where the populations of both rhinoceros and elephants are greatly overlapped.

Our results also showed that *A. thollonii* harbored *Theileria*. cf. *velifera*, which is seemingly maintained in the African buffalo (*Syncerus caffer*) (Yusufmia *et al.*, 2010). In Kenya, *Theileria* cf. *velifera* has been detected in *A. eburneum* nymphs as well as *A. hebraeum* ticks infesting the African buffalo (Mwamuye *et al.*, 2017). This parasite is associated with benign theileriosis in cattle, sheep and goats (Mackenzie & Norval, 1980) whereas its establishment in the elephant and the role of *A. thollonii* in its transmission are both unclear. However, it is likely that the infected young tick stages that feed on livestock may transmit *T. velifera* through transtadial perpetuation to the elephant.

We also identified *Ehrlichia* spp. in the African elephant and the elephant ticks, *A. tholloni* and *R. humeralis* but we did not detect *Anaplasma* spp. in either elephants or ticks. Ehrlichiosis, caused by multiple species including *E. chaffeensis*, *E. ewingii*, *E. canis* and *E. ruminantium*, affects cattle, sheep, goats and dogs. *Ehrlichia ruminantium* has also been linked to Cowdriosis (heart water) disease in the African elephant (Walker & Olwage, 1987). However, this species has not been identified in elephants. The presence of identical and similar haplotypes of *Ehrlichia* in ticks and elephants suggest that *A. thollonii* and *R. humeralis* both with a wide host range are likely to be vectors for *Ehrlichia* spp. transmission between wild and domestic animals. Specifically, transtadial perpetuation of *Ehrlichia* spp. by these three-host ticks, with tick larval and nymph stages of *A. thollonii*

feeding on small domestic ruminants (Mackenzie & Norval, 1980; Gomes, 1993) and adults feeding on elephants, shows that the elephant bont-tick and the elephant could have a role in the epidemiology of Ehrlichiosis especially at the wildlife-livestock interface.

According to MacKenzie and Norval (1980), the heavy infestation of sheep by larvae and nymphs of *A. thollonii* were associated with frequent cases of Heartwater in the Zambezi valley of Zimbabwe. The tick, *R. humeralis* has been found in cattle and camels while among wild animals, it prefers African elephants and sometimes the rhinoceros and African buffalo (Walker *et al.*, 2005; Kariuki *et al.*, 2012; Horak *et al.*, 2017). Its distribution is restricted to Southern Somalia, Eastern Kenya and Northern Tanzania (Walker *et al.*, 2005). In the current study, this tick was present only in the Tsavo elephants. There is no previous record on pathogens harbored by *R. humeralis* or disease association (Walker *et al.*, 2005), thus our study provides the first record on the potential role of the tick species on the epidemiology of *Ehrlichia* spp.

CONCLUSIONS

6. CONCLUSIONS

1st. The main helmint species in Kenya elephants were *Protofasciola*, *Brumptia*, *Murshidia*, *Quilonia* y *Mammomonogamus*.

2nd. Prevalence of trematodes significantly varied across different locations but not social group. However, there was a lack of significant influence of social group and sampling location on prevalence of nematodes.

3rd. The Normalized Difference Vegetation Index is an important driver of variation in eggs elimination across elephant population, because this indice significantly varied across the four habitats and was positively correlated with social groups, being higher in female groups than male social groups.

4th. The morphological and molecular identification of *Amblyomma tholloni* was conclusive based on COX1 gene and inconclusive using the ITS gene.

5th. There is a high genetic diversity across tick populations, which was correlated to the diversity of agro-ecological zones in each sampling locality.

6th. This is the first study of its kind conducted on elephant-tick-pathogen relationships in Kenya and provides a benchmark for evaluating elephant-tick-pathogen relationships elsewhere in the African continent where elephants occur.

7th. This is the first time that *Theileria bicornis* was detected in *Amblyomma thollonii*, in addition *Theileria* cf. *velifera* was also detected in this tick species.

8th. It describes for the first time molecular genetic identification of *Babesia* spp. and *Ehrlichia* spp. in the African elephant. The prevalence of *Babesia* spp. and *Ehrlichia* spp. in ticks was higher than in their elephant hosts suggesting the reservoir status of the ticks for these pathogens. For this reasons more studies are need to unravel the role of this host as reservoirs of tick-borne pathogens.

6. CONCLUSIONES

1ª. Los principales helmintos que afectan a los elefantes en Kenia son *Protofasciola*, *Brumptia*, *Murshidia*, *Quilonia* y *Mammomonogamus*.

2ª. La prevalencia de trematodos varió con la zona de procedencia de los elefantes, pero no con los grupos sociales. Por el contrario, la prevalencia de los nematodos fue similar al considerar la zona y los grupos sociales.

3ª. El índice de vegetación de diferencia normalizada es un importante parámetro para valorar a eliminación de huevos de helmintos en las poblaciones de elefantes, puesto que este índice varió significativamente al considerar las zonas de procedencia de los animales y de los grupos sociales, siendo los integrados por hembras los que presentaron mayor eliminación.

4ª. Con el gen COX1 se demostró que existe concordancia entre la identificación morfológica y molecular de *Amblyomma tholloni* y no se hallaron resultados concluyentes utilizando el gen ITS.

5ª. Existe una gran diversidad genética entre las poblaciones de garrapatas, que se correlacionó con la diversidad de las zonas agroecológicas en cada localidad de muestreo.

6ª. Es el primer estudio realizado sobre las relaciones elefante-garrapata-patógeno en Kenia y proporciona un punto de referencia para otros países del continente africano.

7ª. Es la primera vez que se identifica *Theileria bicornis* en *Amblyomma thollonii*, además, en esta especie de garrapata también se identificó *Theileria* cf. *velifera*.

8ª. Se realizó por primera vez la identificación molecular de *Babesia* spp. y *Ehrlichia* spp. en elefantes africanos. La prevalencia de *Babesia* spp. e *Ehrlichia* spp. en las garrapatas fue mayor que en los elefantes, lo que sugiere que estas podrían estar actuando como reservorio de ambos patógenos, siendo necesario realizar más estudios para determinar el papel de estos hospedadores como reservorios de patógenos transmitidos por garrapatas.

6. CONCLUSIÓNS

1ª. Os principais helmintos que afectan os elefantes en Kenia son *Protofasciola*, *Brumptia*, *Murshidia*, *Quilonia* e *Mammomonogamus*.

2ª. A prevalencia de trematodos variou coa zona de procedencia dos elefantes, pero non cos grupos sociais. Pola contra, a prevalencia dos nematodos foi similar ao considerar a zona e os grupos sociais.

3ª. O índice de vexetación de diferenza normalizada é un importante parámetro para valorar a eliminación de ovos de helmintos nas poboacións de elefantes, posto que este índice variou significativamente ao considerar as zonas de procedencia dos animais e os grupos sociais, sendo os integrados por femias os que presentaron maior eliminación.

4ª. Co xene COX1 demostrouse que existe congruencia entre a identificación morfolóxica e molecular de *Amblyomma tholloni* e non se acharon resultados concluíntes utilizando o xene ITS.

5ª. Existe unha gran diversidade xenética entre as poboacións de carrachas, que se correlacionou coa diversidade das zonas agroecolóxicas en cada localidade de mostraxe.

6ª. É o primeiro estudo realizado sobre as relacións elefante-carracha-patóxeno en Kenia e proporciona un punto de referencia para outros países do continente africano.

7ª. É a primeira vez que se identifica *Theileria bicornis* en *Amblyomma thollonii*, ademais, nesta carracha tamén se achou *Theileria* cf. *velifera*.

8ª. Realízase por primeira vez a identificación molecular de *Babesia* spp. e *Ehrlichia* spp. en elefantes africanos. A prevalencia de *Babesia* spp. e *Ehrlichia* spp. nas carrachas foi maior que nos elefantes, o que suxire que estas poderían estar a actuar como reservorio de ambos patóxenos, sendo necesario realizar máis estudos para determinar o papel destes hospedeiros como reservorios de patóxenos transmitidos por carrachas.

SUMMARY

7. SUMMARY

The African elephant (*Loxodonta Africana*) is the largest land mammal. It is a flagship species that faces multiple and diverse conservation challenges. Both the elephant ranges and demography have continued to decline due to various anthropogenic forces. Currently, the African elephant is listed as a threatened species, specifically due to poaching, which has decimated some populations and contributed to significant decline in demography.

Pathogens that infect elephants are well described, however there is little information on how the parasites interact with the emerging conservation strategies and changing abiotic factors, such as climate and host population isolation.

In this thesis helmint infection patterns, *Amblyomma tholloni* phylogeography and genetic structure and the molecular characterization of *Ehrlichia*, *Anaplasma*, *Babesia* and *Theileria* have been studied.

In relationship with **the patterns of helminth infection** were stablished of age, sex, social structure and Normalized Difference Vegetation Index (NDVI) as primary drivers of infection patterns within and between elephant populations.

Coprological methods were used to identify helminths and to determine infection patterns across different elephant populations in Kenya. Multivariate cluster analyses and measurements of egg dimensions, were used to classify trematode eggs into *Protofasciola* spp. and *Brumptia* sp. and nematode strongyle eggs into *Murshidia*, *Quilonia* and *Mammomonogamus*. Generalized Linear Models and Chi-square analyses were used to test for variation in helminth infection patterns and to identify drivers among elephant populations. The studied drivers were the study location (Tsavo East National Park, Laikipia-Samburu Ecosystem, Maasai Mara National Reserve and Amboseli National Park), age (adults, sub-adults and juveniles), sex, social structure (family and male social groups) and the Normalized difference vegetation index (NDVI).

There was a higher prevalence of nematodes (96.3%) and a 2-fold low prevalence of trematodes (39.1%) in elephants. Prevalence of trematodes significantly varied across different locations (χ^2= 53.13, P < 0.001) but not social group (χ^2 = 0.254, P = 0.614). However, there was a lack of significant influence of social group (χ^2= 1.952, P = 0.162) and sampling location (χ^2= 5.956, P= 0.114) on prevalence of nematodes.

The average NDVI over a 3-month period varied across study locations. The multivariate Generalized Linear Model (GLM) analyses revealed that mean eggs per gram (epg) is positively influenced by a three-month accumulative mean NDVI, and by social group with female social groups having higher epg than male groups. These GLM analyses also revealed that epg varied among elephant population with the Samburu-Laikipia elephants having a significantly higher epg and Tsavo elephants with a lower epg than Amboseli elephants.

Regarding to the **morphological identification of ticks and molecular confirmation of *Amblyomma tholloni*.** The main aim of this research was to determine the possible influence of elephant population on the genetic structure of this tick species in Kenya, using ITS2 as a genetic marker.

Tick genetic analysis from four elephant populations revealed 93 haplotypes from 98 individual ticks based on 826 segregating sites. The Haplotype diversity (Hd) was 0.999 and was similar across host populations. Nucleotide diversity was moderate, 0.108, and nucleotide within populations ranged from 0.022 in Maasai Mara to 0.351 in the Amboseli ecosystem corresponding to the diversity of agro-ecological zones.

Results revealed low (6%) genetic differentiation between the four regions with 94% of molecular genetic variation occurring among ticks within each population, indicating high amounts of gene flow (FST=0.059, P<0.001). All populations of *A. tholloni* in Kenya had a significantly negative Tajima D and Fu & Li's F* and D* suggesting positive selection, genetic hitchhiking, or a recent increase in population size.

However, tests sensitive to demography such as Fu's F_S, Ramos-Onsins & Rozas's R_2 and raggedness index *r* were generally weak suggesting purifying selection. The extensive acaricide use in livestock host of the larval stage of this tick, could be driving purifying selection and genetic hitchhiking of the ITS2 gene.

Agroecological factors influence the genetic diversity of ticks in Kenya while livestock movement shapes the genetic structure of *A. tholloni*. Moreover, the extensive acaricide use in Kenya is likely driving the purifying selection and genetic hitchhiking of ITS2 in ticks.

Finally, a **molecular identification of *Ehrlichia, Anaplasma, Babesia* and *Theileria* in ticks and elephants was carried out.** Although historical records indicate the presence of *Ehrlichia* and *Babesia* in African elephants, not much is known about their prevalence and diversity in elephants and their ticks (*Amblyomma thollonii* and *Rhipicephalus humeralis).*

We amplified and sequenced the hypervariable V4 region of the 18S rRNA gene of *Babesia* and *Theileria* and the heat shock protein gene (groEL) of *Ehrlichia*/*Anaplasma* in DNA extracted from elephant blood (n= 104) and from elephant ticks (n= 52).

Our results showed that the African elephants were infected with a novel *Babesia* spp. while *A. thollonii* was infected with *Theileria bicornis* and *Theileria* cf. *velifera*. This is the first record of *T. bicornis*; a protozoan that is linked to fatal infection in rhinoceros. Elephants and their ticks were all infected with *Ehrlichia* haplotypes (H1-H5) like that identified in Japanese deer.

The prevalence of *Babesia* spp., *Theileria* spp. and *Ehrlichia* spp. in ticks was higher than that of their elephant hosts. About 13.5% of elephants were positive for *Theileria* or *Babesia* while 51% of *A. thollonii* ticks and 27% of *R. humeralis* ticks were positive for *Theileria* or *Babesia*. Moreover, 5.8% of elephants were positive for *Ehrlichia or Anaplasma* compared to 19.5% in *A. thollonii* and 18% in *R. humeralis*. There was no association between the positive result in ticks and their elephant hosts.

Our study reveals that the African elephants are naturally infected with *Babesia* spp. and *Ehrlichia* spp. and opens up an opportunity for further studies to determine the role of elephant as reservoirs of tick-borne pathogens, and to investigate their potential in spreading these pathogens as they range extensively. The presence of *T. bicornis* in *A. thollonii* also suggests a need for experiments to confirm its vector competence.

7. RESUMEN

El elefante africano (*Loxodonta africana*) es el mamífero terrestre más grande. Es una especie emblemática que se enfrenta a múltiples y diversos desafíos de conservación. Tanto las áreas de distribución del elefante como sus poblaciones han disminuido debido a diversas causas antropogénicas. En la actualidad, el elefante africano figura en la lista de especies amenazadas, ya que la caza furtiva que ha diezmado sus poblaciones, contribuyendo a una importante disminución de la demografía.

Los patógenos que infectan a los elefantes están bien descritos, pero hay poca información sobre la forma en que los parásitos interactúan con las nuevas estrategias de conservación y los factores abióticos, como el clima y el aislamiento de las poblaciones de elefantes.

En esta Tesis se han estudiado los patrones de infección por helmintos, la filogeografía y estructura genética de la garrapata *Amblyomma tholloni* y se ha realizado la identificación molecular de *Ehrlichia*, *Anaplasma*, *Babesia* y *Theileria* tanto en los elefantes como en sus garrapatas.

Respecto a la **infección por helmintos**, se utilizaron las técnicas coprológicas para determinar la prevalencia de los helmintos hallados. Basándose en las características morfológicas, se identificaron huevos de los géneros de trematodos *Protofasciola* y *Brumptia* y de los estrongílidos *Murshidia*, *Quilonia* y *Mammomonogamus*.

Se determinó la influencia de diferentes variables: zona de procedencia de los elefantes (Tsavo East National Park, Laikipia-Samburu Ecosystem, Maasai Mara National Reserve y Amboseli National Park), edad (adultos, subadultos y juveniles), sexo, estructura social (machos solitarios y grupos familiares) e Índice de Diferencia Normalizada de la Vegetación (NDVI) sobre los patrones de infección, utilizándose modelos lineales generalizados y la prueba chi-cuadrado.

La prevalencia de nematodos (96,3%) fue superior a la de trematodos (39,1%). La prevalencia de trematodos varió significativamente entre las diferentes zonas (χ^2= 53,13, P < 0,001)

pero no entre los grupos sociales (χ^2 = 0,254, *P* = 0,614). Sin embargo, para los nematodos no existieron diferencias significativas para los grupos sociales (χ^2= 1,952, *P* = 0,162) ni para las zonas de procedencia de los animales (χ^2= 5,956, *P*= 0,114).

Los análisis del Modelo Lineal Generalizado Multivariable (MLGM) revelaron que la media de huevos por gramo (hpg) está influenciada positivamente por una media acumulativa de tres meses de NDVI, y por el grupo social, siendo los grupos sociales formados por hembras los que presentan una carga de huevos mayor. Estos análisis también revelaron que el recuento de hpg variaba entre la población de elefantes, ya que los procedentes de Samburu-Laikipia presentaban una carga de huevos significativamente más alta y los elefantes de Tsavo una carga más baja que los de Amboseli.

Con respecto a la **identificación morfológica de las garrapatas y posterior confirmación molecular de *Amblyomma tholloni*.** El principal objetivo de esta investigación fue determinar la posible influencia de la población de elefantes en la estructura genética de la garrapata *A. tholloni* en Kenia, utilizando el gen ITS2 como marcador genético.

Mediante el análisis genético de las garrapatas de las cuatro poblaciones de elefantes permitió detectar 93 haplotipos en 98 garrapatas individuales basados en 826 sitios de segregación. La diversidad de haplotipos fue de 0,999 y siendo similar en todas las poblaciones de hospedadores. La diversidad de nucleótidos era moderada, 0,108, oscilando entre 0,022 en Maasai Mara y 0,351 en el ecosistema de Amboseli, lo que sugiere una diversidad genética entre las distintas zonas agroecológicas.

Los resultados revelaron una baja (6%) diferenciación genética entre las cuatro regiones, con un 94% de variación genética molecular entre las garrapatas dentro de cada población, lo que indica un alto flujo genético (FST=0,059, P<0,001). Todas las poblaciones de *A. tholloni* en Kenya presentaron un Tajima D y unas F* y D* de Fu & Li, significativamente negativas lo que puede indicar una selección positiva, arrastre por ligamento o un aumento reciente del tamaño de la población de garrapatas.

Sin embargo, las pruebas sensibles a la demografía, como el FS de Fu, el R2 de Ramos-Onsins & Rozas y el índice de irregularidad r, fueron en general débiles, lo que sugiere una selección purificadora. El uso extensivo de acaricidas contra el estadio larvario de esta garrapata sobre el ganado hospedador podría estar impulsando la selección purificadora y el arrastre por ligamento del gen ITS2.

Con este estudio podemos concluir que los factores agroecológicos influyen en la diversidad genética de *A. tholloni* en Kenya, mientras que el movimiento del ganado conforma la estructura genética de esta garrapata. Además, es probable que el uso extensivo de acaricidas en Kenya esté impulsando la selección purificadora y el arrastre por ligamento del gen ITS2 en las garrapatas.

Finalmente se realizó una **identificación molecular de *Ehrlichia, Anaplasma, Babesia* y *Theileria* en los elefantes y en sus garrapatas.** Aunque previamente ya se había detectado *Ehrlichia* y *Babesia* en los elefantes africanos, no existen muchos estudios de prevalencia y caracterización genética de estos patógenos en los elefantes y sus garrapatas (*Amblyomma thollonii* y *Rhipicephalus humeralis).*

Se realizó una extracción de ADN a partir de la sangre de 104 elefantes y de 52 garrapatas. Posteriormente, se amplificó y secuenció la región hipervariable V4 del gen 18S del ARNr de *Babesia* y *Theileria* y el gen de la proteína de choque térmico (*groEL*) de *Ehrlichia*/*Anaplasma*.

Nuestros resultados mostraron que los elefantes africanos estaban infectados con una nueva especie de *Babesia* por el contrario en la garrapata *A. thollonii* se detectó *Theileria bicornis* y *Theileria* cf. *velifera*. Este es el primer registro de *T. bicornis*, protozoo que, previamente había provocado una infección mortal en rinocerontes. En los elefantes y en sus garrapatas se detectaron 5 haplotipos de *Ehrlichia* (H1-H5) que previamente se había hallado en ciervos japoneses.

La prevalencia de *Babesia* spp., *Theileria* spp. y *Ehrlichia* spp. en las garrapatas fue mayor que en los elefantes; puesto que, el 13,5% de los elefantes fueron positivos a *Theileria* o *Babesia*, mientras que el

51% de *A. thollonii* y el 27% de *R. humeralis* fueron positivas a *Theileria* o *Babesia*. Además, el 5,8% de los elefantes fueron positivos para *Ehrlichia* o *Anaplasma* frente al 19,5% hallado en *A. thollonii* y al 18% en *R. humeralis*. No obstante, no se encontró una asociación entre las garrapatas positivas a los patógenos estudiados y sus elefantes.

Nuestro estudio revela que los elefantes africanos están infectados naturalmente con *Babesia* spp. y *Ehrlichia* spp. y abre la oportunidad de realizar más estudios para determinar el papel de estos hospedadores como reservorios de patógenos transmitidos por garrapatas, e investigar su rol en la propagación de estos y otros patógenos. La detección de *T. bicornis* en *A. thollonii* también sugiere la necesidad de realizar más estudios que permitan determinar su competencia como vector.

7. RESUMO

O elefante africano (*Loxodonta africana*) é o mamífero terrestre máis grande que actualmente existe na terra. É unha especie emblemática que se enfronta a múltiples e diversos desafíos de conservación. Na actualidade, tanto as áreas de distribución do elefante como as súas poboacións diminuíron debido a diversas causas antropoxénicas.

Actualmente, o elefante africano figura na lista de especies ameazadas, xa que a caza furtiva que ha decimado as súas poboacións, contribuíndo a unha importante diminución da demografía. O sistema de sociedade dos elefantes é de tipo fusión-fisión fluído no que machos e femias viven en esferas separadas pero superpostas. As familias ou mandas están formadas por femias emparentadas e as súas crías (entre 2 e 30) de diferentes idades, dirixidas pola femia de maior idade, á que se dá o nome de *matriarca*. En ocasións, as mandas de femias poden fusionarse durante un tempo, chegando a incluír centos de individuos. Os machos abandonan a familia das femias cando son adolescentes e forman grupos con outros animais da súa idade, para posteriormente levar unha vida solitaria, achegándose normalmente ás mandas de femias soamente durante a época de celo. Con todo, os elefantes machos tampouco se afastan en exceso da súa familia, e recoñécena perfectamente cando volven atopala. As infeccións e enfermidades que afectan os elefantes, en xeral, estudáronse pouco, debido a que, ata o de agora, críase que as enfermidades, especialmente, as de etioloxía parasitarias, non tiñan consecuencias negativas sobre as poboacións de animais salvaxes, entre as que se inclúen as dos elefantes africanos. Ademais, a maioría das investigacións de céntranse principalmente na taxonomía dos parasitos e hai moi poucos estudos sobre a forma en que os parasitos interactúan coas novas estratexias de conservación e os factores abióticos, como o clima e o illamento das poboacións de elefantes.

Debido aos escasos estudos epidemiolóxicos que existen sobre a epidemioloxía das infeccións parasitarias nos elefantes, nesta Tese

propuxémonos analizar a influencia das zonas agro ecolóxicas, da estrutura social e do Índice de Diferenza Normalizada da Vexetación sobre a **infección por helmintos**. Os elefantes de 4 zonas de procedencia dos elefantes que son as que albergan as maiores poboacións destes paquidermos en Quenia e que non teñen conectividade entre se. O Parque Nacional Tsavo East (TENP) está situado en pártea sueste de Quenia, é unha sabana semiárida que experimenta un patrón de choiva anual bimodal con choivas longas que ocorren en abril-maio, mentres que as choivas curtas ocorren en novembro-decembro. A área ten 7.727 elefantes segundo o informe do censo de 2018 do Servizo de Vida Silvestre de Quenia. O ecosistema Laikipia-Samburu (LSE) atópase na rexión central de Quenia. O censo de elefantes de 2017 realizado polo Servizo de Vida Silvestre de Quenia estima 7.166 elefantes. É un pasteiro de sabana árida con precipitacións bimodales, que caen principalmente en abril e novembro. A Reserva Nacional Maasai Mara (MMNR) atópase en pártea sur de Quenia ao longo da fronteira entre Quenia e Tanzania, contigua ao Serengeti. En xeral, a área é unha extensa sabana de pasteiros. O censo de elefantes de 2017 informou de 2.493 elefantes neste hábitat. O Parque Nacional Amboseli (ANP) está situado na base do Monte Kilimanjaro en pártea sur de Quenia. Esta área ten 2.127 elefantes. É unha terra de pasteiros abertos de sabana seca xeralmente árida mesturada con parches de matogueiras e bosques de *Acacia xanthophloea*. Unha rede de marismas resultante de recárgaa subterránea do derretemento do nevado do monte Kilimanjaro proporciona auga permanente. Así mesmo considerouse que, un grupo social feminino era o que estaba formado por femias e a súa descendencia, mentres que un grupo social masculino definiuse como un macho solitario ou un grupo de dous machos vistos de preto o momento da observación. Utilizouse o Índice de Diferenza Normalizada da Vexetación (NDVI) que é unha medida da reflectancia e absorbancia da vexetación nos espectros infravermello e azul da vexetación verde. En xeral, o NDVI está fortemente influenciado por parámetros climáticos como a temperatura de precipitación e a humidade do chan. Estes factores inflúen directa ou

indirectamente na relación hospedado-parasito e na transmisión dos helmintos con fases larvarias de vida libre (nematodos gastrointestinais).

Recolléronse birlos de feces frescas que se abriron con coidado e en cada análise coprolóxico empregouse aproximadamente 20 gramos de feces. Utilizáronse as técnicas coprolóxicas de flotación, sedimentación e McMaster. Examináronse un total de 243 mostras de feces; 71 de machos solitarios ou grupos de machos independentes e 172 de grupos de femias. Se muestrearon 62 grupos de familias na Reserva Nacional de Maasai Mara, 37 do Parque Nacional de Tsavo East, 27 do Parque Nacional de Amboseli e 19 do Ecosistema de Laikipia-Samburu. Ademais, analizáronse 19 mostras de machos de MMNR, 22 do TENP, 16 do ANP e 14 do LSE.

A prevalencia de nematodos foi elevada (96,3%) e non se acharon diferenzas significativas ao considerar os grupos sociais ($\chi2 = 1,952$, $P = 0,162$), nin as zonas de procedencia dos animais ($\chi2 = 5,956$, $P= 0,114$).

A prevalencia de eliminación de ovos de trematodos foi do 39,1% e variou significativamente entre as diferentes zonas ($\chi2 = 53,13$, $P < 0,001$) probablemente debido ás diferenzas que existen nesas zonas respecto a abundancia de carafio que actúan como hospedeiros intermediarios deses trematodos. Pola contra, non se acharon diferenzas na prevalencia de eliminación ao considerar os grupos sociais ($\chi2 = 0,254$, $P = 0,614$). Ademais, na nosa análise, observamos unha variación significativa entre poboacións na prevalencia, que se debeu principalmente á prevalencia de trematodos.

As análises do Modelo Lineal Xeneralizado Multivariable (MLGM) revelaron que a media de ovos por gramo está influenciada positivamente por unha media acumulativa de tres meses de NDVI, e polo grupo social, sendo os grupos sociais formados por femias os que presentan unha carga de ovos maior. Estas análises tamén revelaron que o reconto de ovos por gramo variaba entre a poboación de elefantes, xa que os procedentes de Samburu-Laikipia presentaban unha carga de ovos significativamente máis alta e os elefantes de Tsavo East unha carga máis baixa que os de Amboseli.

Utilizando as medidas de lonxitude e anchura medias dos ovos realizouse unha análise de mestura finita gaussiana para agrupar os ovos de nematodos e trematodos en unidades taxonómicas operativas (OTU). Este modelo revelou que as poboacións de elefantes en Quenia estaban infectadas con trematodos que pertencían a 2 OTUs, o OTU1 correspondíase con ovos de *Protofasciola robusta* e o OTU2 con ovos de *Brumptia bicaudata*. Para os nematodos, o modelo indicou 5 OTUs, un grupo do xénero *Murshidia* (OTU1) e 3 grupos que pertencen ao xénero *Quilonia* (OTU2, OTU3 e OTU4) e un grupo do OTU5 que se correspondeu con *Mammomonogamus loxodontis*.

Para obter **as carrachas que afectan os elefantes e realizar a súa posterior identificación morfolóxica**, foi necesario inmobilizar aos animais. Para iso, obtívose a autorización do Comité de Investigación e Ética do Servizo de Vida Silvestre de Quenia (KWS/BRM/5001) que é a institución encargada de protexer e conservar a vida silvestre neste país. As mostras obtivéronse durante as intervencións programadas (tratamento clínico e translocacións) e participaron veterinarios de vida silvestre con experiencia que seguiron os protocolos e pautas aprobados sobre a práctica veterinaria de vida silvestre de 2006 e a Lei de Cirurxiáns Veterinarios de Quenia.

As carrachas obtivéronse da orella e a trompa dos elefantes e conserváronse en botes nitróxeno líquido que se etiquetaban coa referencia do animal. Unha vez no laboratorio, as carrachas se desconxelaron e identificaron, baixo un microscopio estereoscópico, seguindo as diversas claves morfolóxicas (Olwage, 1987; Horak *et al*., 2018).

Colleitáronse un total de 698 carrachas: 242 eran de 10 elefantes do Parque Nacional de Tsavo East, 162 procedían de 4 elefantes do Ecosistema de Laikipia-Samburu, 100 carrachas eran de 16 animais da Reserva Nacional de Maasai Mara e 97 eran de 9 elefantes do Parque Nacional de Amboseli.

Identificáronse as especies *Amblyomma tholloni, Amblyomma gemma, Rhipicephalus praetextatus, Rhipicephalus humeralis* e *Rhipicephalus appendiculatus*. A especie predominante foi *A. tholloni* (62%), seguida de *Rhipicephalus praetextatus* (35%) e en moito

menor porcentaxe acháronse *R. appendiculatus* (2%), *R. humeralis* (1%) e *A. gemma* (0,5%). *Amblyomma tholloni* achouse nos elefantes das 4 zonas agro ecolóxicas, polo contrario, *Rhipicephalus praetextatus* non se observou nos animais da Reserva Nacional de Maasai Mara, mentres que *R. appendiculatus* e *R. humeralis* só obtívose dos elefantes do Parque Nacional de Tsavo East e *A. gemma* dos do Ecosistema de Laikipia-Samburu.

Debido a que *Amblyomma tholloni* foi a carrachas máis prevalente nos elefantes das 4 zonas de mostraxe, propuxémonos estudar a posible influencia da poboación de elefantes na estrutura xenética desta especie de carracha; así como establecer os patróns de estrutura xeográfica en *A. tholloni* debido ao illamento do hospedeiro e examinar as inferencias da demografía histórica das carrachas e as forzas evolutivas que impulsan a diversidade xenética.

Para lograr este obxectivo analizouse unha submostra de 137 carrachas identificadas como *A. tholloni*. Para facilitar a extracción de ADN, estas carrachas conxeláronse individualmente en nitróxeno líquido en tubos de 1,5 ml e se homoxenizaron usando micromorteros (Sigma Aldrich, Missouri, United States). Posteriormente, realizouse a extracción de ADN utilizando o kit comercial DNeasy (QIAGEN, Hilden, Alemaña) seguindo o protocolo recomendado polo fabricante para a extracción de ADN procedente de sangue e tecidos.

Para comprobar que as carrachas foran correctamente identificadas morfoloxicamente, realizouse un protocolo de PCR destinado a amplificar un fragmento de 650 pares de base (pb) do xene COX1 mitocondrial das carrachas utilizando os cebador directo LepF1 (5'-ATTCAACCAATCATAAAGATATTGG-3') e inverso LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-3').

Para realizar a análise xenética de poboacións de *A. tholloni*, estas mesmas mostras sometéronse a un protocolo de PCR para amplificar un fragmento de 1500 pb do xene ITS2 utilizando o cebador directo F1-ITS2 (5′-CGAGACTTGGTGTGAATTGCA-3′) e inverso (R1-ITS2 5′-TCCCATACACCACATTTCCCG-3′).

Ambos os protocolos de PCR realizáronse nun volume total de 25 µl constituído por 1 µl do ADN de móstralas problema, 12,5 µl dun

preparado comercial onde se incluían os reactivos de PCR (OneTaq® Quick-Loade®, 2X con tampón estándar New England Biolabs-NEB, Massachusetts, EE.UU.) e 0,5 µl de cada un dos cebadores directo e inverso a unha concentración 10 mm. Como control negativo, utilizouse auga de grao molecular libre de nucleasas. A amplificación levou a cabo nun termociclador SimpliAmp (Life Technologies) seguindo as seguintes condicións: unha desnaturalización inicial a 94ºC durante 1 min seguida de 35 ciclos de desnaturalización a 94ºC durante 20 segundos, un aliñamento a 46ºC durante 20 segundos e 1 min de extensión a 68ºC. A extensión final foi a 68ºC durante 5 min.

Os fragmentos obtidos tras a realización da reacción de amplificación sometéronse a unha electroforese horizontal en xeles de agarosa ao 1% nun tampón TAE 1x, aplicando unha voltaxe de 90 V durante 35 min. As bandas de ADN evidenciáronse mediante tintura con bromuro de etidio e visualizáronse cun transiluminador UV Gelpilot 100 bp plus (Qiagen, Alemaña). Todos os amplicones co tamaño de banda esperado enviáronse para a súa secuenciación a Macrogen Europe B.V.

As secuencias obtidas visualizáronse e corrixiron empregando o software Geneious v11 (Kearse *et al.*, 2012). As secuencias das carrachas consenso para os xenes ITS2 e COX1 xeráronse a partir de datos de secuencia directa e inversa e exportáronse como arquivos fasta a MEGA X (Kumar *et al.*, 2018) e DNAsp 6 (Rozas *et al.*, 2017) para unha mellor aliñación e análise.

Das 137 *A. tholloni* extraídas, púidose amplificar e secuenciar o xene ITS2 de 98. Ademais, púidose secuenciar un subconxunto de 6 mostras de carrachas para o xene COX1 obténdose 5 haplotipos (T1, T3, T4, T5, T6).

Os resultados de ITS2 BLAST mostraron que todas as secuencias das carrachas deste estudo tiñan unha coincidencia de secuencia do 97-98% cunha *Amblyomma* sp. previamente identificada na Reserva Nacional de Maasai Mara.

A análise xenética de *A. tholloni* das catro poboacións de elefantes permitiu detectar 93 haplotipos en 98 carrachas individuais baseados en 826 sitios de segregación. A diversidade de haplotipos foi

de 0,999 e sendo similar en todas as poboacións de hospedeiros. A diversidade de nucleótidos era moderada (0,108) oscilando entre 0,022 en Maasai Mara e 0,351 no ecosistema de Amboseli, o que suxire unha diversidade xenética entre as distintas zonas agroecolóxicas.

Os resultados revelaron unha baixa (6%) diferenciación xenética entre as catro rexións, cun 94% de variación xenética molecular entre as carrachas dentro de cada poboación, o que indica un alto fluxo xenético (FST= 0,059, P<0,001). Todas as poboacións de *A. tholloni* en Quenia presentaron un Tajima D e unhas F* e D* de Fu & Li, significativamente negativas o que pode indicar unha selección positiva, arrastre por ligamento ou un aumento recente do tamaño da poboación de carrachas.

Con todo, as probas sensibles á demografía, como o FS de Fu, o R2 de Ramos-Onsins & Rozas e o índice de irregularidade r, foron en xeral débiles, o que suxire unha selección purificadora. O uso extensivo de acaricidas contra o estadio larvario de *A. tholloni* ou sobre o gañado hospedeiro podería estar a impulsar a selección purificadora e o arrastre por ligamento do xene ITS2.

Finalmente realizouse unha **identificación molecular de *Ehrlichia, Anaplasma, Babesia* e *Theileria* nos elefantes e nas seus carrachas**. Aínda que previamente xa se detectou *Ehrlichia* e *Babesia* nos elefantes africanos, non existen moitos estudos de prevalencia e caracterización xenética destes patóxenos nos elefantes e as seus carrachas (*Amblyomma thollonii* e *Rhipicephalus humeralis*). Por iso, propuxémonos determinar a presenza de hemoparásitos e a prevalencia destes en elefantes e carrachas colleitadas dos mesmos hospedeiros procedentes de diferentes áreas, con obxecto de estudar a epidemioloxía destes patóxenos nos elefantes e identificar o papel de *A. thollonii* no mantemento e propagación da enfermidade.

A extracción de ADN realizouse a partir de 200 µl de sangue de 104 elefantes conservada en tubos con EDTA e de 52 carrachas. Para iso empregouse o kit comercial DNeasy (QIAGEN, Hilden, Alemaña) e seguíronse os protocolos descritos polo fabricante para a extracción de ADN a partir de sangue e tecido. As carrachas procesáronse como se sinalou anteriormente. Posteriormente, se amplificou e secuenciou a

rexión hipervariable V4 do xene 18 S do ARNr de *Babesia* spp. e *Theileria* spp. e o xene da proteína de choque térmico (groEL) de *Ehrlichia* spp./*Anaplasma* spp.

Para a detección de *Theileria* spp. e *Babesia* spp., realizouse unha PCR aniñada para a amplificación dun segmento de 450-500 pb do xene que codifica para a subunidade 18 S do ARN ribosómico. A primeira reacción de PCR levouse a cabo empregando o xogo de cebadores directo e inverso: ILO-9029 (5' CGGTAATTCCAGCTCCAATAGCGT-3') e ILO-9030 (5'-TTTCTCTCAAAGGTGCTGAAGGAGT-3'), mentres que para a segunda reacción de PCR empregouse o xogo de cebadores ILO-9029 (5'-CGGTAATTCCAGCTCCAATAGC-3') e ILO-7782 (5'-AACTGACGACCTCCAATCTCTAGTC-3'). Os oligonucleótidos utilizados neste estudo sintetizáronse en Macrogen Inc., Europa.

A primeira reacción de PCR realizouse nun volume total de 10 µl que consistiu en 5 µl de HotStarTaq Master Mix (QIA-XENE, Hilden, Alemaña), 0,5 µl de 10 pmol/ul de cada un dos cebadores directo e inverso, 2 µl do ADN problema e 2 µl de auga PCR estéril. As condicións empregadas nesta reacción de PCR incluíron unha desnaturalización inicial a 95°C durante 15 minutos; seguida de 30 ciclos de desnaturalización (95°C durante 30 segundos), aliñamento (55°C durante 30 segundos) e extensión (72°C durante 1 minuto); seguidos dunha extensión final a 72°C durante 5 minutos nun termociclador T100 (Bio-Rad). O volume da segunda reacción de PCR foi de 25 µl constando de 0,5 µl de cada un dos cebadores, 1,0 µl do produto de PCR obtido na primeira reacción, 10,5 µl de auga de PCR estéril e 12,5 µl de HotStarTaq Master Mix (QIAGEN, Hilden, Alemaña). As condicións de ciclo utilizadas foron idénticas ás empregadas na primeira reacción de PCR. En ambas as reaccións de PCR empregouse un control positivo, unha mostra positiva de *Theileria parva* obtida dun traballo previo no laboratorio e auga PCR estéril como control negativo para as amplificacións.

Para o estudo de *Ehrlichia* e *Anaplasma* se amplificou un fragmento de 200-300 pb que codifica para o xene groESL (Park *et al.*, 2005). Na primeira reacción de amplificación empregouse o xogo

de cebadores EF1 (5'-CTG AYG GTA TGC AGT TTG-3' e ER2 (5'-AYR YYT TTA GCA GTA CC-3') e na segunda os cebadores EF3 (5'-GGT ATG CAG TTT GAI CG-3') e ER4 (5'-TCT TTT CTY CTR TCA CC-3').

A primeira reacción de PCR realizouse nun volume total de 10 µl que consistiu en 1 µl de ADN de cada unha das mostras, 0,5 µl a unha concentración 10 pmol/µ l de cada un dos cebadores, 5 µl do preparado OneTaq1 Quick-Loade1 2X Master Mix con tampón estándar (New England Bio-labs-NEB, Massachusetts, USA) e 2 µl de auga destilada libre de nucleasas. A primeira amplificación realizouse seguindo o seguinte protocolo: desnaturalización inicial, 94ºC durante 1 minuto; 20 ciclos de desnaturalización (94ºC durante 20 segundos), aliñamento (50ºC durante 20 segundos) e extensión (68ºC durante 30 segundos). Finalmente someteuse a unha extensión final de 68ºC durante 5 minutos. Todas as reaccións leváronse a cabo nun termociclador T100 thermal cycler (Bio-Rad). A segunda reacción de PCR realizouse nun volume total de 25 µl que consistía en 1,0 µl do produto da primeira reacción de PCR, 10,5 µl de auga destilada e 12,5 µl dun preparado comercial (HotStarTaq Master Mix, QIAGEN, Hilden, Alemaña). As condicións de PCR empregadas foron as mesmas que as descritas para a primeira reacción de PCR.

En ambas as reaccións empregouse unha mostra positiva a *Anaplasma* spp. como control positivo e auga destilada como control negativo.

Os fragmentos obtidos tras a realización da reacción de amplificación sometéronse a unha electroforeses horizontal en xeles de agarosa ao 1,5% nun tampón TAE 1x, aplicando unha voltaxe de 90 V durante 35 min. As bandas de ADN evidenciáronse mediante tintura con bromuro de etidio e visualizáronse cun transiluminador UV Gelpilot 100 bp plus (Qiagen, Alemaña). Todos os produtos de PCR positivos se purificaron e secuenciaron en Macrogen Inc., Europa, tanto en dirección directa como inversa.

Para estudar os haplotipos, as secuencias obtidas foron comparadas coas previamente depositadas no GenBank (Benson *et*

al., 2009), para iso utilizouse o algoritmo BLASTn (Altschul *et al.*, 1990).

Todas as secuencias de *Theileria* e *Babesia* editadas deste estudo depositáronse en GenBank co número de acceso MN595045-MN595058 e todas as secuencias de *Ehrlichia* depositáronse en GenBank cos números de acceso, MN602332-MN602336.

As secuencias aliñáronse usando MUSCLE v. 3.8.31 (Edgar, 2004) en MEGA X por separado para cada un dos patóxenos estudados (*Theileria, Babesia* e *Ehrlichia/Anaplasma*). O mellor modelo de evolución de secuencias e heteroxeneidade para as secuencias específicas aliñadas estimouse utilizando MEGA X (Tamura *et al.*, 2013). Para realizar as árbores filoxenéticos de *Babesia* spp. e *Theileria* spp. empregouse o modelo de substitución de nucleótidos de 2 parámetros de Kimura (Kimura, 1980) cunha distribución gamma discreta para modelar as diferenzas na taxa de evolución entre os sitios (*Babesia*, G = 0,469; *Theileria*, G = 0,204). Permitiuse que a variación da taxa para algúns sitios fose evolutivamente invariable nos modelos de evolución de secuencias de *Babesia* (I = 34,6%) e *Theileria* (I = 47,1% do sitio). Pola contra, para *Ehrlichia* o mellor modelo para a realización do estudo filoxenético foi o modelo de 3 parámetros de Tamura (Kimura, 1981) cunha distribución gamma discreta para as diferenzas na taxa de evolución entre os sitios (5 categorías (+ G, parámetro = 0,209).

Os nosos resultados mostraron que os elefantes en Quenia estaban infectados cunha nova especie de *Babesia*, mentres que na carracha *A. thollonii* detectouse *Theileria bicornis* e *Theileria* cf. *velifera*. Este é o primeiro rexistro de *T. bicornis* nos elefantes de Quenia, sen embargo, este protozoo previamente provocara unha infección mortal en rinocerontes. Ademais, nos elefantes e nas súas carrachas detectáronse 5 haplotipos de *Ehrlichia* (H1-H5) que previamente se achou en cervos xaponeses.

A prevalencia de *Babesia* spp., *Theileria* spp. e *Ehrlichia* spp. nas carrachas foi maior que nos elefantes, posto que, o 13,5% dos elefantes foron positivos a *Theileria* ou *Babesia*, mentres que o 51% de *A. thollonii* e o 27% de *R. humeralis* foron positivas a *Theileria* ou

Babesia. Ademais, o 5,8% dos elefantes foron positivos para *Ehrlichia* ou *Anaplasma* fronte ao 19,5% achado en *A. thollonii* e ao 18% en *R. humeralis*. Con todo, non se atopou unha asociación entre as carrachas positivas aos patóxenos estudados e os seus elefantes.

O noso estudo revela que os elefantes en Quenia están infectados naturalmente con *Babesia* spp. e *Ehrlichia* spp. e abre a oportunidade de realizar máis estudos para determinar o papel destes hospedeiros como reservorios de patóxenos transmitidos por carrachas, e investigar o seu rol na propagación destes e outros patóxenos. A detección de *T. bicornis* en *A. thollonii* tamén suxire a necesidade de realizar máis estudos que permitan determinar a competencia desta carracha como vector.

REFERENCES

8. REFERENCES

Abe, E. (1982). The status of elephants in Uganda: Queen Elizabeth National Park. *Pachyderm***,** 15, 49-52.

Agnew, A.D.Q. (1968). Observations on the changing vegetation of Tsavo National Park (East). *African Journal of Ecology,* 6 (1), 75-80.

Aguirre, E. (1969). Evolutionary history of the elephant. *Science***,** 164(3886), 1366-1376.

Albayrak, S. (2008). Use of reflectance measurements for the detection of N, P, K, ADF and NDF contents in sainfoin pasture. *Sensors,* 8 (11), 7275-7286.

Albery, G.F., Kenyon, F., Morris, A., Morris, S., Nussey, D.H., Pemberton, J. M. (2018). Seasonality of helminth infection in wild red deer varies between individuals and between parasite taxa. *Parasitology,* 145 (11), 1410-1420.

Alexandre, D.Y. (1978). Le rôle disséminateur des éléphants en forêt de Tai, Côte-d'Ivoire. *La Terre et la vie*.

Altizer, S., Harvell, D., Friedle, E. (2003). Rapid evolutionary dynamics and disease threats to biodiversity. *Trends in Ecology & Evolution*, 18 (11), 589-596.

Altschul, S.F., Gish, W., Miller, W., Myers, E. W., Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215 (3), 403-410.

Amici, R.R. (2001). The history of Italian parasitology. *Veterinary Parasitology*, 98(1-3), 3-30.

Amzati, G.S., Pelle, R., Muhigwa, J.B.B., Kanduma, E.G., Djikeng, A., Madder, M., Marcotty, T. (2018). Mitochondrial phylogeography and population structure of the cattle tick *Rhipicephalus appendiculatus* in the African Great Lakes region. *Parasites & Vectors,* 11(1), 329.

Andrew, H.R., & Norval, R.A.I. (1989). The carrier status of sheep, cattle and African buffalo recovered from heart water. *Veterinary Parasitology*, 34 (3), 261-266.

Apio A, Plath M, Wronski T. (2006). Patterns of gastrointestinal parasitic infections in the bushbuck *Tragelaphus scriptus* from the Queen Elizabeth National Park, Uganda. *Journal of Helminthology,* 80: 213-8.

Archie, E.A., Chiyo, P.I. (2012). Elephant behavior and conservation: social relationships, the effects of poaching, and genetic tools for management. *Molecular Ecology*, 21 (3), 765-778.

Archie EA, Moss CJ, Alberts SC. (2006 a). The ties that bind: genetic relatedness predicts the fission and fusion of social groups in wild African elephants. *Proceedings of the Royal Society B: Biological Sciences,* 273:5 13-22.

Archie, E.A., Morrison, T.A., Foley, C.A., Moss, C.J., Alberts, S.C. (2006 b). Dominance rank relationships among wild female African elephants, *Loxodonta africana*. *Animal Behaviour*, 71 (1), 117-127.

Arneberg, P., Skorping, A., Grenfell, B., Read, A.F. (1998). Host densities as determinants of abundance in parasite communities. **P***roceedings of the Royal Society of London. Series B: Biological Sciences,* 26 5(1403), 1283-1289.

Babes, V. (1888). Sur l'hemoglobinurie bacterienne du boeuf. *Comptes Rendus de l' Academie des Sciences* 107, 692-694.

Baines, L., Morgan, E.R., Ofthile, M., Evans, K. (2015). Occurrence and seasonality of internal parasite infection in elephants, *Loxodonta africana*, in the Okavango Delta, Botswana. *International Journal for Parasitology: Parasites and Wildlife,* 4 (1), 43-48.

Barnes, R.F.W. (1982). Mate searching behaviour of elephant bulls in a semi-arid environment *Animal Behaviour,* 30 (4), 1217-1223.

Barrett, L.G., Thrall, P.H., Burdon, J.J., Linde, C.C. (2008). Life history determines genetic structure and evolutionary potential of host–parasite interactions. *Trends in Ecology & Evolution,* 23 (12), 678-685.

Barriel, V., Thuet, E., Tassy, P. (1999). Molecular phylogeny of Elephantidae. Extreme divergence of the extant forest African elephant. *Comptes Rendus de l'Académie des Sciences-Series III-Sciences de la Vie,* 322 (6), 447-454.

Baskaran, N., Balasubramanian, M., Swaminathan, S., Desai, A.A. (1995). Home range of elephants in the Nilgiri Biosphere Reserve, south India. A week with elephants. *Bombay Natural History Society and Oxford University Press, Bombay***,** 296-313.

Beati, L., Patel, J., Lucas-Williams, H., Adakal, H., Kanduma, E.G., Tembo-Mwase, E., Kelly, P. (2012). Phylogeography and demographic history of *Amblyomma variegatum* (Fabricius)

(Acari: Ixodidae), the tropical bont tick. *Vector-Borne and Zoonotic Diseases*, 12 (6), 514-525.

Bekker, C.P., Vink, D., Pereira, C.M.L., Wapenaar, W., Langa, A., Jongejan, F. (2001). Heartwater (*Cowdria ruminantium* Infection) as a Cause of Postrestocking Mortality of Goats in Mozambique. *Clinical and Diagnostic Laboratory Immunology*, 8 (4), 843-846.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. (2009). GenBank. 2009. *Nucleic Acids Research,* 37, D26-D31.

Binetruy, F., Buysse, M., Lejarre, Q., Barosi, R., Villa, M., Rahola, N., ... & Duron, O. (2020). Microbial community structure reveals instability of nutritional symbiosis during the evolutionary radiation of Amblyomma ticks. *Molecular Ecology*, *29*(5), 1016-1029.

Brocklesby, D. W., Campbell, H. (1963). A Babesia of the African elephant. *African Journal of Ecology*, 1 (1), 119-119.

Brown, W.C., Palmer, G. H. (1999). Designing blood-stage vaccines against *Babesia bovis* and *B. bigemina*. *Parasitology Today*, 15 (7), 275-281.

Brumfitt, K.N. (2015). Assessment of body condition of African elephants *(Loxodonta Africana)* in northeast of Etosha national park, Namibia: How it relates to strongyle parasite egg counts and nutritional value of *feed* . Doctoral dissertation, University of Namibia). http://hdl.handle.net/11070/1535.

Bryant, A.S., Hallem, E.A. (2018). Temperature-dependent behaviors of parasitic helminths. *Neuroscience Letters,* 687, 290-303.

Buma, B. (2015). Disturbance interactions: characterization, prediction, and the potential for cascading effects. *Ecosphere*, 6 (4), 1-15.

Burridge, M.J., Simmons, L.A., Allan, S.A. (2000). Introduction of potential heartwater vectors and other exotic ticks into Florida on imported reptiles. *Journal of Parasitology*, 86 (4), 700-704.

Burroughs, R.E., Penzhorn, B.L., Wiesel, I., Barker, N., Vorster, I., Oosthuizen, M.C. (2017). Piroplasms in brown hyaenas (*Parahyaena brunnea*) and spotted hyaenas (*Crocuta crocuta*) in Namibia and South Africa are closely related to Babesia lengau. *Parasitology Research*, 116 (2), 685-692.

Bowman, D. D., & Georgi, J. R. (2003). Georgis' parasitology for veterinarians/Parasitology for veterinarians Georgis' parasitology for veterinarians.

Camus, E., Barre, N., Martinez, D., Uilenberg, G., Deem, S. L. (1996). Heartwater (Cowdriosis): A Review, 2nd edn. *Parasitology Today*, 12 (8), 328.

Caron, A., Morand, S., & de Garine-Wichatitsky, M. (2012). Epidemiological interaction at the wildlife/livestock/human interface: can we anticipate emerging infectious diseases in their hotspots? A framework for understanding emerging diseases processes in their hot spots. In *New frontiers of molecular epidemiology of infectious diseases* (pp. 311-332). Springer, Dordrecht.

Carreno, R.A., Kinsella, J.M. (2008). Equinurbia blakei n. sp. (Nematoda: Strongyloidea: Strongylidae) from the African

forest elephant, *Loxodonta cyclotis*. *Comparative Parasitology*, 75 (2), 196-199.

Casati, S., Bernasconi, M. V., Gern, L., Piffaretti, J.C. (2008). Assessment of intraspecific mtDNA variability of European *Ixodes ricinus* sensu stricto (Acari: Ixodidae). *Infection, Genetics and Evolution*, 8 (2), 152-158.

Chase, M.J., Schlossberg, S., Griffin, C.R., Bouch., P.J.C., Djene, S.W., Elkan, P.W., Sutcliffe, R. (2016). Continent-wide survey reveals massive decline in African savannah elephants. *Peer J*, 4, e2354.

Chitimia, L., Lin, R.Q., Cosoroaba, I., Braila, P., Song, H. Q., Zhu, X.Q. (2009). Molecular characterization of hard and soft ticks from Romania by sequences of the internal transcribed spacers of ribosomal DNA. *Parasitology Research*, 105 (4), 907-911.

Chiyo, P.I., Archie, E.A., Hollister-Smith, J.A., Lee, P.C., Poole, J.H., Moss, C.J., Alberts, S.C. (2011). Association patterns of African elephants in all-male groups: the role of age and genetic relatedness. *Animal Behaviour*, 81 (6), 1093-1099.

Chiyo, P.I., Moss, C.J., Alberts, S.C. (2012). The influence of life history milestones and association networks on crop-raiding behavior in male African elephants. *PloS One*, 7 (2), e31382.

Condy, J.B. (1974). Observations on internal parasites in Rhodesian elephant, *Loxodonta Africana* Blumenbach 1797. *Proceedings and Transactions of the Rhodesia Scientific Association.* Diss. med. vet. Zürich 1974.

Connors, V.A., Nickol, B.B. (1991). Effects of *Plagiorhynchus cylindraceus* (Acanthocephala) on the energy metabolism of adult starlings, *Sturnus vulgaris*. *Parasitology*, 103 (3), 395-402.

Coulson, G., Cripps, J. K., Garnick, S., Bristow, V., Beveridge, I. (2018). Parasite insight: assessing fitness costs, infection risks and foraging benefits relating to gastrointestinal nematodes in wild mammalian herbivores. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 373 (1751), 20170197.

Crampton, P.L., Gichanga, M.M. (1979). A survey of resistance to acaricides in economically important Ixodidae (Acari) of the major cattle-raising areas of Kenya. *Bulletin of Entomological Research*, 69 (3), 427-439.

Criado-Fornelio, A., González-del-Río, M.A., Buling-Saraña, A., Barba-Carretero, J. C. (2004). The "expanding universe" of piroplasms. *Veterinary Parasitology*, 119 (4), 337-345.

Cripps, J., Beveridge, I., Martin, J.K., Borland, D., Coulson, G. (2015). Temporal dynamics of helminth infections in eastern grey kangaroos (*Macropus giganteus*) in Victoria. *Australian Journal of Zoology*, 63 (3), 163-174.

Criscione, C. D., Poulin, R., Blouin, M.S. (2005). Molecular ecology of parasites: elucidating ecological and microevolutionary processes. *Molecular Ecology*, 14 (8), 2247-2257.

Croft, B.A., McGroarty, D.L. (1973). A model study of acaricide resistance, spider mite outbreaks, and biological control

patterns in Michigan apple orchards**.** *Environmental Entomology*, 2 (4), 633-638.

Cumming, D. H. M. (1982). The influence of large herbivores on savanna structure in Africa. In *Ecology of tropical savannas* (pp. 217-245). Springer, Berlin, Heidelberg.

Cutullé, C., Jonsson, N.N., Seddon, J. (2009). Population structure of Australian isolates of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus***.** *Veterinary Parasitology*, 161 (3-4), 283-291.

Davenport, M.L., Nicholson, S.E. (1993). On the relation between rainfall and the Normalized Difference Vegetation Index for diverse vegetation types in East Africa. *International Journal of Remote Sensing*, 14 (12), 2369-2389.

Debeila, E.M. (2013). *Occurrence of Anaplasma and Ehrlichia species in African buffalo (Syncerus caffer) in Kruger National Park and Hluhluwe-iMfolozi Park in South Africa*. Doctoral Dissertation, University of Pretoria). http://hdl.handle.net/2263/25111.

Debruyne, R. (2005). A case study of apparent conflict between molecular phylogenies: the interrelationships of African elephants. *Cladistics*, 21 (1), 31-50.

de la Fuente, J., Almazán, C., Van Den Bussche, R.A., Bowman, J., Yoshioka, J.H., Kocan, K. M. (2005). Characterization of genetic diversity in *Dermacentor andersoni* (Acari: Ixodidae) with body size and weight polymorphism. *Experimental Parasitology*, 109 (1), 16-26.

De Meeus T, Be´Ati L, Delaye C, Aeschlimann A, Renaud FO (2012) Sex-biased genetic structure in the vector of lyme disease, Ixodes ricinus. Evolution 56(9):1802–1807

de Waal, D.D. (2000). GLOBAL IMPORTANCE OF PIROPLASMOSIS. *The* **Journal of Protozoology.**

de Waal, D.T., Van Heerden, J. (2004). Equine Babesiosis. In du Plessis, I. *Infectious Diseases of Livestock,* 425-434.

Dinnis, R.E., Seelig, F., Bormane, A., Donaghy, M., Vollmer, S.A., Feil, E.J., Margos, G. (2014). Multilocus sequence typing using mitochondrial genes (mtMLST) reveals geographic population structure of *Ixodes ricinus* ticks. *Ticks and Tick-borne Diseases,* 5 (2), 152-160.

Dipeolu, O.O. (1976). The occurrence of ticks on a baby African elephant in Nigeria. **African Journal of Ecology,** 14(3), 227-227.

Douglas-Hamilton, I. (1972). African Elephant Ivory Trade Study: Final Report (Excerpts). *Elephant*, 1 (4), 11.

Douglas-Hamilton, I. (1972). On the ecology and behaviour of the African elephant: *"the elephants of Lake Manyara"*.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rurangirwa, F. (2001). Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia,* descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia*

phagocytophila. *International Journal of Systematic and Evolutionary Microbiology*, 51 (6), 2145-2165.

Edgar, R.C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics,* 5 (1), 113.

Eisen, R.J., Kugeler, K.J., Eisen, L., Beard, C.B., Paddock, C.D. (2017). Tick-borne zoonoses in the United States: persistent and emerging threats to human health. *ILAR Journal,* 58(3), 319-335.

Eisenberg, J.F., McKay, G.M., Jainudeen, M.R. (1971). Reproductive behavior of the Asiatic elephant (*Elephas maximus maximus* L.). *Behaviour,* 193-225.

Eltringham, S.K. (1977). The numbers and distribution of elephant *Loxodonta africana* in the Rwenzori National Park and Chambura Game Reserve, Uganda. *African Journal of Ecology,* 15 (1), 19-39.

Eltringham, S.K., Malpas, R.C. (1980). The decline in elephant numbers in Rwenzori and Kabalega Falls National Parks, Uganda. *African Journal of Ecology,* 18 (1), 73-86.

Evans, K.E., Harris, S. (2008). Adolescence in male African elephants, *Loxodonta africana*, and the importance of sociality. *Animal Behaviour***,** 76 (3), 779-787.

Excoffier, L., Lischer, H.E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources,* 10 (3), 564-567.

Excoffier, L., Smouse, P.E., Quattro, J.M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131 (2), 479-491.

Eygelaar, D., Jori, F., Mokopasetso, M., Sibeko, K. P., Collins, N. E., Vorster, I., Oosthuizen, M.C. (2015). Tick-borne haemoparasites in African buffalo (*Syncerus caffer*) from two wildlife areas in Northern Botswana. *Parasites & Vectors*, 8 (1),26.

Ezenwa, V.O. (2004). Host social behavior and parasitic infection: a multifactorial approach. *Behavioral Ecology***,** 15 (3), 446-454.

Faburay, B., Jongejan, F., Taoufik, A., Ceesay, A., Geysen, D. (2008). Genetic diversity of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks and small ruminants in The Gambia determined by restriction fragment profile analysis. *Veterinary Microbiology*, 126 (1-3), 189-199.

Farrar, T. J., Nicholson, S. E., & Lare, A. R. (1994). The influence of soil type on the relationships between NDVI, rainfall, and soil moisture in semiarid Botswana. II. NDVI response to soil oisture. *Remote sensing of Environment*, *50*(2), 121-133.

Felstenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39(4),783-791.

Fowler, M., Mikota, S. K. (2006). *Biology, Medicine, and Surgery of Elephants*. (Eds.). *John Wiley & Sons.*

Froeschke, G., Harf, R., Sommer, S., Matthee, S. (2010). Effects of precipitation on parasite burden along a natural climatic gradient in southern Africa–implications for possible shifts in

infestation patterns due to global changes. *Oikos*, 119 (6), 1029-1039.

Fu, Y.X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, 147 (2), 915-925.

Fu, Y.X., Li, W. H. (1993). Statistical tests of neutrality of mutations. *Genetics*, 133 (3), 693-709.

Funk, S.M. (2001). The role of disease in carnivore ecology and conservation. *Carnivore Conservation*.

Gadd, M.E. (2005). Conservation outside of parks: attitudes of local people in Laikipia, Kenya. *Environmental Conservation*, 50-63.

Gamon, J.A., Field, C.B., Goulden, M.L., Griffin, K.L., Hartley, A.E., Joel, G., Valentini, R. (1995). Relationships between NDVI, canopy structure, and photosynthesis in three Californian vegetation types. *Ecological Applications*, 5 (1), 28-41.

Ganswindt, A., Heistermann, M., Hodges, K. (2005). Physical, physiological, and behavioral correlates of musth in captive African elephants (*Loxodonta africana*). *Physiological and Biochemical Zoology*, 78 (4), 505-514.

Ganswindt, A., Rasmussen, H.B., Heistermann, M., Hodges, J.K. (2005). The sexually active states of free-ranging male African elephants (*Loxodonta africana*): defining musth and non-musth using endocrinology, physical signals, and behavior. *Hormones and Behavior*, 47 (1), 83-91.

Gassó, D., Feliu, C., Ferrer, D., Mentaberre, G., Casas-Díaz, E., Velarde, R., ... & Lavín, S. (2015). Uses and limitations of

faecal egg count for assessing worm burden in wild boars. *Veterinary Parasitology*, *209*(1-2), 133-137.

Gheerbrant, E. (1998). The oldest known proboscidean and the role of Africa in the radiation of modem orders of placentals. *Bulletin of the Geological Society of Denmark*, 44 (2), 181-185.

Goldenberg, S.Z., de Silva, S., Rasmussen, H.B., Douglas-Hamilton, I., Wittemyer, G. (2014). Controlling for behavioural state reveals social dynamics among male African elephants, *Loxodonta africana*. *Animal Behaviour*, 95, 111-119.

Gooding, R.H. (1996). Genetic variation in arthropod vectors of disease-causing organisms: obstacles and opportunities. *Clinical Microbiology Reviews*, 9(3), 301-320.

Gassó, D., Feliu, C., Ferrer, D., Mentaberre, G., Casas-Díaz, E., Velarde, R., Lavín, S. (2015). Uses and limitations of faecal egg count for assessing worm burden in wild boars. *VeterinaryParasitology*, 209 (1-2), 133137.

Gomes, A.F. (1993). The tick vectors of cowdriosis in Angola. *Revue d'élevage et de Médecine Vétérinaire des pays Tropicaux*, 46 (1-2), 237-243.

Govender, D., Oosthuisen, M.C., Penzhorn, B.L. (2011). Piroplasm parasites of white rhinoceroses (*Ceratotherium simum*) in the Kruger National Park, and their relation to anaemia. *Journal of the South African Veterinary Association*, 82 (1), 36-40.

Green, D.S., Roloff, G.J., Heath, B.R., Holekamp, K.E. (2015). Temporal dynamics of the reponses by African mammals to prescribed fire. *The Journal of Wildlife Management*, 79 (2), 235-242.

Grossman, C.J. (1985). Interactions between the gonadal steroids and the immune system. *Science*, 227(4684), 257-261.

Groves, C.P., Grubb, P. (2000). Do *Loxodonta cyclotis* and *L. africana* interbreed? *Elephant***,** 2 (4), 4.

Grubb, P., Groves, C.P., Dudley, J. P., Shoshani, J. (2000). Living African elephants belong to two species: *Loxodonta africana* (Blumenbach, 1797) and *Loxodonta cyclotis* (Matschie, 1900). *Elephant*, 2 (4), 3.

Guerra-Silveira, F., Abad-Franch, F. (2013). Sex bias in infectious disease epidemiology: patterns and processes. *PloS one*, 8 (4), e62390.

Guglielmone, A.A., Nava, S., Mastropaolo, M., Mangold, A.J. (2013). Distribution and genetic variation of *Amblyomma triste* (Acari: Ixodidae) in Argentina. *Ticks and tick-borne diseases*, 4 (5), 386-390.

Gunathilaka, N., Niroshana, D., Amarasinghe, D., Udayanga, L. (2018). Prevalence of gastrointestinal parasitic infections and assessment of deworming program among cattle and buffaloes in Gampaha District, Sri Lanka. *BioMed Research International*, 2018.

Gunn, A., Irvine, R.J. (2003). Subclinical parasitism and ruminant foraging strategies: a review. *Wildlife Society Bulletin*, 117-126.

Hall-Martin, A.J. (1987). Role of musth in the reproductive strategy of the African elephant (*Loxodonta africana*). *South African Journal of Science*, 83 (10), 616-620.

Hamer, S. A., Bernard, A. B., Donovan, R. M., Hartel, J. A., Wrangham, R. W., Otali, E., & Goldberg, T. L. (2013). Coincident tick infestations in the nostrils of wild chimpanzees and a human in Uganda. *The American Journal of Tropical Medicine and Hygiene*, *89*(5), 924-927.

Hanks, J. (1969). Growth in weight of the female African elephant in Zambia. *African Journal of Ecology*, 7 (1), 7-10.

Harpending, H.C. (1994). Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology*, 591-600.

Hausfater, G., Meade, B.J. (1982). Alternation of sleeping groves by yellow baboons (*Papio cynocephalus*) as a strategy for parasite avoidance. *Primates*, 23 (2), 287-297.

Hawkins, E., Kock, R., McKeever, D., Gakuya, F., Musyoki, C., Chege, S. M., Skilton, R.A. (2015). Prevalence of *Theileria equi* and *Babesia caballi* as well as the identification of associated ticks in sympatric Grevy's zebras (**Equus grevyi**) and donkeys (*Equus africanus asinus*) in northern Kenya. *Journal of Wildlife Diseases*, 51 (1), 137-147.

Hawthorne, W.D., Parren, M.P. (2000). How important are forest elephants to the survival of woody plant species in Upper Guinean forests?. *Journal of Tropical Ecology***,** 133-150

Haynes, G. (1993). Mammoths, mastodonts, and elephants*: biology, behavior and the fossil record*. Cambridge University Press.

Hebert, P.D., Penton, E.H., Burns, J.M., Janzen, D.H., Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes*

fulgerator. *Proceedings of the National Academy of Sciences***,** 101 (41), 14812-14817.

Hersh, M.H., Tibbetts, M., Strauss, M., Ostfeld, R.S., Keesing, F. (2012). Reservoir competence of wildlife host species for *Babesia microti*. *Emerging Infectious Diseases,* 18 (12), 1951.

Hilburn, L.R., Sattler, P.W. (1986). Are tick populations really less variable and should they be?. *Heredity,* 57 (1), 113-117.

Höfle, U., Vicente, J., Nagore, D., Hurtado, A., Pena, A., de La Fuente, J., Gortazar, C. (2004). The risks of translocating wildlife: pathogenic infection with *Theileria* sp. and *Elaeophora elaphi* in an imported red deer. *Veterinary Parasitology,* 126 (4), 387-395.

Hollister-Smith, J. A., Poole, J. H., Archie, E. A., Vance, E. A., Georgiadis, N. J., Moss, C. J., & Alberts, S. C. (2007). Age, musth and paternity success in wild male African elephants, *Loxodonta africana*. *Animal Behaviour*, *74*(2), 287-296.

Hoogstral H. (1956). African Ixodoidea. 1. Ticks of the Sudan. *Research Report NM 005 05029 07***.** Department of the Navy, Bureau of Medicine and Surgery Washington, DC.

Horak, I. G., Williams, E.J. (1986). Parasites of domestic and wild animals in South Africa. XVIII. The crowned guinea fowl (*Numida meleagris*), an important host of immature ixodid ticks. *The Onderstepoort Journal of Veterinary Research,* 53 (2), 119-122.

Horak, I. G., Matthee, S. (2003). Parasites of domestic and wild animals in South Africa. XLIII. Ixodid ticks of domestic dogs

and cats in the Western Cape Province. *The Onderstepoort Journal of Veterinary Research*, 70 (3), 187.

Horak, I.G., Heyne, H., Donkin, E.F. (2010). Parasites of domestic and wild animals in South Africa: XLVIII. Ticks (Acari: Ixodidae) infesting domestic cats and wild felids in southern Africa. *Onderstepoort Journal of Veterinary Research*, 77, 01-07.

Horak, I. G., Boshoff, C. R., Cooper, D. V., Foggin, C. M., Govender, D., Harrison, A., ... & Nel, P. J. (2017). Parasites of domestic and wild animals in South Africa. XLIX. Ticks (Acari: Ixodidae) infesting white and black rhinoceroses in southern Africa. *Onderstepoort Journal of Veterinary Research*, *84*(1), 1-11.

Horak, I.G., Heyne, H., Williams, R., Gallivan, G.J., Spickett, A.M., Bezuidenhout, J.D., Estrada-Peña, A. (2018). *The ixodid ticks (Acari: Ixodidae) of southern Africa*. Ed. Springer.

Jongejan, F., Uilenberg, G. (2004). The global importance of ticks. *Parasitology-Cambridge*, 129, S3.

Kagaruki, L.K. (1991). Tick (Acari: Ixodidae) resistance to organochlorine acaricides in Tanzania. *International Journal of Pest Management*, 37 (1), 33-36.

Kakoma, I., Mehlhorn, H. (1994). *Parasitic Protozoa*. 2nd ed. Kreier J. P. **7** 1993 141 216. Academic Press San Diego, Calif

Kangwana, K.F. (1993). *Elephants and Maasai: conflict and conservation in Amboseli, Kenya*. Doctoral Dissertation. University of Cambridge).

Kariuki, E.K., Penzhorn, B.L., Horak, I.G. (2012). Ticks (Acari: Ixodidae) infesting cattle and African buffaloes in the Tsavo conservation area, Kenya: research communication. *Onderstepoort Journal of Veterinary Research*, 79 (1), 1-4.

Kariuki, E., Kutima, H., Kock, M., Horak, I.G., Jooste, R., Neves, L. (2019). Ixodid ticks (Acari: Ixodidae) collected from African savanna elephants (*Loxodonta africana*) and African forest elephants (*Loxodonta cyclotis*). *Onderstepoort Journal of Veterinary Research,* 86 (1), 1-5.

Kasari, T.R., Miller, R.S., James, A.M., Freier, J.E. (2010). Recognition of the threat of *Ehrlichia ruminantium* infection in domestic and wild ruminants in the continental United States. *Journal of the American Veterinary Medical Association***,** 237 (5), 520-530.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Thierer, T. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28 (12), 1647-1649.

Keesing, F., Allan, B.F., Young, T.P., Ostfeld, R.S. (2013). Effects of wildlife and cattle on tick abundance in central Kenya. *Ecological Applications,* 23 (6), 1410-1418.

Kempf, F., De Meeûs, T., Vaumourin, E., Noel, V., Taragel'ová, V., Plantard, O., McCoy, K.D. (2011). Host races in *Ixodes ricinus*, the European vector of Lyme borreliosis. *Infection, Genetics and Evolution,* 11(8), 2043-2048.

Ketchum, H. R., Teel, P. D., Coates, C. J., Strey, O. F., & Longnecker, M. T. (2014). Genetic variation in 12S and 16S mitochondrial rDNA genes of four geographically isolated populations of Gulf Coast ticks (Acari: Ixodidae). *Journal of medical entomology*, *46*(3), 482-489.

Kim, J.H., Choi, M.H., Bae, Y.M., Oh, J.K., Lim, M.K., Hong, S.T. (2011). Correlation between discharged worms and fecal egg counts in human clonorchiasis. *PLoS Neglected Tropical Diseases*, 5(10), e1339.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16 (2), 111-120.

Kimura, M. (1981). Estimation of evolutionary distances between homologous nucleotide sequences. *Proceedings of the National Academy of Sciences*, 78 (1), 454-458.

King'ori, E.M., Obanda, V., Ndambiri, E.M., Runo, S.M., Chiyo, P.I. (2018). Adding injury to infection: The relationship between injury status and genetic diversity of *Theileria* infecting plains zebra, *Equus quagga*. *Infection, Genetics and Evolution*, 58, 269-278.

King'ori, E., Obanda, V., Chiyo, P. I., Soriguer, R. C., Morrondo, P., Angelone, S. (2019). Molecular identification of *Ehrlichia*, *Anaplasma*, *Babesia* and *Theileria* in African elephants and their ticks. *PLOS ONE*, 14 (12), e0226083.

King'ori, E., Obanda, V., Chiyo, P.I., Soriguer, R.C., Morrondo, P., Angelone, S. (2020). Patterns of helminth infection in Kenyan elephant populations. *Parasites Vectors*, 13: 145.

Kinsella, J.M., Deem, S.L., Blake, S., Freeman, A.S. (2004). Endoparasites of African forest elephants (*Loxodonta africana cyclotis*) from the Republic of Congo and Central African Republic. *Comparative Parasitology*, 71 (2), 104-110.

Kioko, J., Kiringe, J., Omondi, P. (2006). Human-elephant conflict outlook in the Tsavo-Amboseli ecosystem, Kenya. *Pachyderm*, 41 (41), 53-61.

Kjemtrup, A.M., Conrad, P.A. (2000). Human babesiosis: an emerging tick-borne disease. *International Journal for Parasitology*, 30 (12-13), 1323-1337.

Knapp, S.E., Krecek, R.C., Horak, I.G., Penzhorn, B.L. (1997). Helminths and arthropods of black and white rhinoceroses in southern Africa. *Journal of Wildlife Diseases*, 33 (3), 492-502.

Koch, R. (1904). Rhodesian investigations. *Cape Agricultural Journal*, 24, 33-45.

Kock, N.D., van Vliet, A.H., Charlton, K., Jongejan, F. (1995). Detection of *Cowdria ruminantium* in blood and bone marrow samples from clinically normal, free-ranging Zimbabwean wild ungulates. *Journal of Clinical Microbiology*, 33 (9), 2501-2504.

Kovalev, S.Y., Golovljova, I.V., Mukhacheva, T.A. (2016). Natural hybridization between *Ixodes ricinus and Ixodes persulcatus*

ticks evidenced by molecular genetics methods. *Ticks and tick-borne diseases,* 7 (1), 113-118.

Krakowetz, C.N., Dergousoff, S.J., Chilton, N.B. (2010). Genetic variation in the mitochondrial 16S rRNA gene of the American dog tick, *Dermacentor variabilis* (Acari: Ixodidae). *Journal of Vector Ecology,* 35 (1), 163-173.

Krakowetz, C.N., Lindsay, L.R., Chilton, N.B. (2011). Genetic diversity in *Ixodes scapularis* (Acari: Ixodidae) from six established populations in Canada. *Ticks and Tick-borne Diseases,* 2 (3), 143-150.

Krakowetz, C.N., Lindsay, L.R., Chilton, N.B. (2014). Genetic variation in the mitochondrial 16S ribosomal RNA gene of *Ixodes scapularis* (Acari: Ixodidae). *Parasites & Vectors,* 7 (1), 530.

Krasnov, B.R., Bordes, F., Khokhlova, I.S., Morand, S. (2012). Gender-biased parasitism in small mammals: patterns, mechanisms, consequences. *Mammalia,* 76 (1), 1-13.

Krecek, R.C., Reinecke, R.K., Horak, I.G. (1989). Internal parasites of horses on mixed grassveld and bushveld in Transvaal, Republic of South Africa. *Veterinary Parasitology,* 34 (1-2), 135-143.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution,* 35 (6), 1547-1549.

Kumsa, B., Signorini, M., Teshale, S., Tessarin, C., Duguma, R., Ayana, D., Cassini, R. (2014). Molecular detection of

piroplasms in ixodid ticks infesting cattle and sheep in western Oromia, Ethiopia. *Tropical Animal Health and Production,* 46 (1), 27-31.

Kuttler, K. L. (1988). World-wide impact of babesiosis. *Babesiosis of Domestic Animals and Man.*, 1-22.

Lack, J.B., Reichard, M.V., Van Den Bussche, R.A. (2012). Phylogeny and evolution of the Piroplasmida as inferred from 18S rRNA sequences. *International Journal for Parasitology*, 42 (4), 353-363.

Lampo, M., Rangel, Y., Mata, A. (1998). Population genetic structure of a three-host tick, *Amblyomma dissimile*, in eastern Venezuela. *The Journal of Parasitology*, 1137-1142.

Langbauer, W.R., Payne, K.B., Charif, R.A., Rapaport, L., Osborn, F. (1991). African elephants respond to distant playbacks of low-frequency conspecific calls. *Journal of Experimental Biology*, 157 **(**1), 35-46.

Latrofa, M. S., Dantas-Torres, F., Annoscia, G., Cantacessi, C., & Otranto, D. (2013). Comparative analyses of mitochondrial and nuclear genetic markers for the molecular identification of *Rhipicephalus* spp. *Infection, Genetics and Evolution*, *20*, 422-427.

Lausen, L., Bekoff, M. (1978). *Loxodonta Africana: Mammalian Species 92*. Published by American Society of Mammalogists,1-8.

Laws, R. M. (1967). Eye lens weight and age in African elephants. *African Journal of Ecology*, 5 (1), 46-52.

Laws, R.M. (1969). Aspects of reproduction in the African elephant, *Loxodonta africana*. *Journal of Reproductive Fertility*, 6, 193-217.

Laws, R. M. (1970). Elephants as agents of habitat and landscape change in East Africa. *Oikos*, 1-15.

Laws, R. M. (1970). Elephants and habitats in north Bunyoro, Uganda. *African Journal of Ecology*, *8*(1), 163-180.

Laws, R.M., Parker, I.S.C. (1968). Recent studies on elephant populations in East Africa. *Symposium Zoology Society London*, 21, pp. 319-359).

Laws, R.M., Parker, I.S.C., Johnstone, R.C.B. (1974). *Elephants and their habitats: the ecology of elephants in North Bunyoro, Uganda*. Clarendon Press.

Laws, R.M., Parker, I.S., Johnstone, R.C. (1975). *Elephants and their habitats*. Clarendon Press.

Lee, P.C. (1986). Early social-development among African elephant calves. *National Geographic Research*, 2 (3), 388-401.

Lee, P.C. (1987). Allomothering among African elephants. *Animal Behaviour*, 35 (1), 278-291.

Lee, P. C. (1989). Family structure, communal care and female reproductive effort. *Comparative socioecology. The behaviour and ecology of humans and other mammals*, 323-340.

Lee, P.C., Moss, C.J. (1986). Early maternal investment in male and female African elephant calves. *Behavioral Ecology and Sociobiology*, 18 (5), 353-361.

Lee, P.C., Moss, C.J. (1995). Statural growth in known-age African elephants (*Loxodonta africana*). *Journal of Zoology*, 236 (1), 29-41.

Leggett, K.E. (2006). Home range and seasonal movement of elephants in the Kunene Region, northwest Namibia. *African Zoology*, 41 (1), 173.

Leimgruber, P., McShea, W.J., Brookes, C.J., Bolor-Erdene, L., Wemmer, C., Larson, C. (2001). Spatial patterns in relative primary productivity and gazelle migration in the Eastern Steppes of Mongolia. *Biological Conservation*, 102 (2), 205-212.

Levine, N.D. (1971). Taxonomy of the piroplasms. *Transactions of the American Microscopical Society*, 2-33.

Levine, N. D. (1988). Blood parasites: the piroplasms. *The protozoan phylum Apicomplexa*, *2*, 35-45.

Levine, N.D., Corliss, J.O., Cox, F.E.G., Deroux, G., Grain, J., Honigberg, B.M., Merinfeld, E.G. (1980). A Newly Revised Classification of the Protozoa* The Committee on Systematics Evolution of the Society of Protozoologists. *The Journal of Protozoology*, 27 (1), 37-58.

Li, J., Lewis, J., Rowland, J., Tappan, G., Tieszen, L.L. (2004). Evaluation of land performance in Senegal using multi-temporal NDVI and rainfall series. *Journal of Arid Environments*, 59 (3), 463-480.

Li, Z.B., Cheng, T.Y., Xu, X.L., Song, L.L., Liu, G.H. (2017). Genetic variation in mitochondrial genes of the tick *Haemaphysalis*

flava collected from wild hedgehogs in China. *Experimental and Applied Acarology*, 71 (2), 131-137.

Librado, P., Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25 (11), 1451-1452.

Liu, X., Chen, Z., Ren, Q., Luo, J., Xu, X., Wu, F., Liu, G. (2018). Genetic diversity of *Haemaphysalis qinghaiensis* (Acari: Ixodidae) in western China. *Experimental and Applied Acarology*, 74 (4), 427-441.

Lodge, D.M., Brown, K.M., Klosiewski, S.P., Stein, R.A., Covich, A. P., Leathers, B.K., Bronmark, C. (1987). Distribution of freshwater snails: spatial scale and the relative importance of physicochemical and biotic factors. *American Malacological Bulletin*, 5 (1), 73-84.

Lopez-Velez, R., Palomar, A. M., Oteo, J. A., Norman, F. F., Pérez-Molina, J. A., & Portillo, A. (2015). Novel *Candidatus rickettsia* species detected in nostril tick from human, Gabon, 2014. *Emerging infectious diseases*, *21*(2), 325.

Lourens, J.H.M., Tatchell, R.J. (1979). Studies on acaricide resistance in *Rhipicephalus evertsi evertsi* Neumann (Acarina: Ixodidae) in East Africa. Identification and inheritance of a resistance factor to organochlorines. *Bulletin of Entomological Research*, 69 (2), 235-242.

MacKenzie, P.K.I., Norval, R. A. I. (1980). The transmission of *Cowdria ruminantium* by *Amblyomma tholloni*. *Veterinary Parasitology*, 7 (3), 265-268.

MacPhee, R. D. E., & Flemming, C. (1999). Requiem Aeternam. In *Extinctions in near time* (pp. 333-371). Springer, Boston, MA.

Magnusson, A., Skaug, H., Nielsen, A., Berg, C., Kristensen, K., Maechler, M., ... & Brooks, M. E. (2017). glmmTMB: generalized linear mixed models using template model builder. *R package version 0.1*, *3*.

Mans, B.J., Pienaar, R., Latif, A.A. (2015). A review of *Theileria* diagnostics and epidemiology. *International Journal for Parasitology: Parasites and Wildlife*, 4 (1), 104-118.

Martin, R.B. (1978). Aspects of elephant social organization. *Rhodesia Science News*, 12 (8), 184-187.

Martínez-Guijosa, J., Martínez-Carrasco, C., López-Olvera, J. R., Fernández-Aguilar, X., Colom-Cadena, A., Cabezón, O., Garel, M. (2015). Male-biased gastrointestinal parasitism in a nearly monomorphic mountain ungulate. *Parasites & Vectors*, 8 (1), 165.

Masangane, F.S.W., Mbati, P.A., Malatje, S.M. (2004). A survey of parasitic helminths in equines and communal farms in the NE Free State province in South Africa. *Journal of the South African Veterinary Association*, 76, 177.

Matsumoto, K., Parola, P., Rolain, J. M., Jeffery, K., Raoult, D. (2007). Detection of "*Rickettsia* sp. strain Uilenbergi" and" *Rickettsia* sp. strain Davousti" in *Amblyomma tholloni* ticks from elephants in Africa. *BMC Microbiology*, 7 (1), 74.

Mazé-Guilmo, E., Blanchet, S., McCoy, K. D., Loot, G. (2016). Host dispersal as the driver of parasite genetic structure: a paradigm lost?. *Ecology Letters*, 19 (3), 336-347.

Mbaya, A.W., Nwosu, C.O., Aliyu, M.M., Ahmed, T.A. (2006). A comparative study of gastrointestinal parasites of captive and free living wild animals in the semi-arid zone of North-eastern Nigeria. *Nigerian Journal of Experimental and Applied Biology*, 1, 59-63.

Mbaya, A.W., Aliyu, M.M., Nwosu, C.O., Ibrahim, U.I. (2008). Effect of Berenil® and Cymelarsan® on the alteration of biochemical parameters in red-fronted gazelles (*Gazella rufifrons*) experimentally infected with *Trypanosoma brucei*. *Revue d'élevage et de Médecine Vétérinaire des Pays Tropicaux*, 61 (3-4).

Mbaya, A.W., Ogwiji, M., Kumshe, H.A. (2013). Effects of host demography, season and rainfall on the prevalence and parasitic load of gastrointestinal parasites of free-living elephants (*Loxodonta africana*) of the Chad Basin National Park, Nigeria. *Pakistan Journal of Biological Sciences: PJBS*, 16 (20), 1152-1158.

McComb, K., Moss, C., Durant, S. M., Baker, L., Sayialel, S. (2001). Matriarchs as repositories of social knowledge in African elephants. *Science***,** 292 (5516), 491-494.

McCoy, K.D., Boulinier, T., Tirard, C., Michalakis, Y. (2003). Host-dependent genetic structure of parasite populations: differential dispersal of seabird tick host races. *Evolution*, 57 (2), 288-296.

McKay, G.M. (1973). Behavior and ecology of the Asiatic elephant in southeastern Ceylon. *Smithsonian Contributions to Zoology*.

McKenna, P.B. (1981). The diagnostic value and interpretation of faecal egg counts in sheep. *New Zealand Veterinary Journal*, 29 (8), 129-132.

McLean, E. R., Kinsella, J. M., Chiyo, P., Obanda, V., Moss, C., & Archie, E. A. (2012). Genetic identification of five strongyle nematode parasites in wild African elephants (*Loxodonta africana*). *Journal of Wildlife Diseases*, *48*(3), 707-716.

MacPhee, R.D. Sues, H.D. (1999). *Extinctions in near time*, 2. Springer Science & Business Media.

Mehlhorn, H., Schein, E. (1998). Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. *Parasitology Research,* 84 (6), 467-475.

Mehlhorn, H., Schein, E., Voigt, W.P. (1980). Light and electron microscopic study on developmental stages of *Babesia canis* within the gut of the tick *Dermacentor reticulatus*. *The Journal of Parasitology*, 220-228.

Mehlhorn, H., & Schein, E. (1985). The piroplasms: life cycle and sexual stages. In *Advances in parasitology* (Vol. 23, pp. 37-103). Academic Press.

Mehlhorn, H., Schein, E., Ahmed, J.S. (1994). *Theileria*. In: *Parasitic Protozoa*, 217-304. Academic Press.

Merz, G. (1986). Movement patterns and group size of the African forest elephant *Loxodonta africana cyclotis* in the Tai National Park, Ivory Coast. *African Journal of Ecology*, 24 (2), 133-136.

Mihalca, A.D., Cozma, V., Şuteu, E., Marinculic, A., Boireau, P. (2010). The quest for piroplasms: from Babeş and Smith to molecules. *Sciences Parasitology***,** 11, 14-19.

Miller, D., Jackson, B., Riddle, H.S., Stremme, C., Schmitt, D., Miller, T. (2015). Elephant (*Elephas maximus*) health and management in Asia: variations in veterinary perspectives. *Veterinary Medicine International***,** 2015.

Mills, E.C., Poulsen, J.R., Fay, J.M., Morkel, P., Clark, C.J., Meier, A., White, L.J. (2018). Forest elephant movement and habitat use in a tropical forest-grassland mosaic in Gabon. *PloS One*, 13 (7), e0199387.

Mixson, T.R., Lydy, S.L., Dasch, G.A., Real, L.A. (2006). Inferring the population structure and demographic history of the tick, *Amblyomma americanum* Linnaeus. *Journal of Vector Ecology*, 31 (1), 181-192.

Mixson, T. R., Campbell, S. R., Gill, J. S., Ginsberg, H. S., Reichard, M. V., Schulze, T. L., & Dasch, G. A. (2006). Prevalence of *Ehrlichia*, *Borrelia*, and *Rickettsial* agents in *Amblyomma americanum* (Acari: Ixodidae) collected from nine states. *Journal of medical entomology*, *43*(6), 1261-1268.

Morgan, E.R., Van Dijk, J. (2012). Climate and the epidemiology of gastrointestinal nematode infections of sheep in Europe. *Veterinary Parasitology*, 189 (1), 8-14.

Moss, C.J. (1977) The Amboseli Elephants. Wildlife News 12(2), 9-12

Moss, C.J. (1981). Social circles. *Wildlife News*, 16 (1), 2-7.

Moss, C.J. (1983). Oestrous behaviour and female choice in the African elephant. *Behaviour*, 86 (3-4), 167-195.

Moss, C.J. (1988). *Elephant Memories: Thirteen Years in the Life of an Elephant Family* E. William Morrow, New York.

Moss, C.J. (2001). The demography of an African elephant (*Loxodonta africana*) population in Amboseli, Kenya. *Journal of Zoology***,** 255 (2), 145-156.

Moss, C.J., Poole, J.H. (1983). Relationships and social structure of African elephants. *Primate Social Relationships: an integrated approach***,** 315, 325.

Mueller, T., Olson, K. A., Fuller, T. K., Schaller, G. B., Murray, M. G., Leimgruber, P. (2008). In search of forage: predicting dynamic habitats of Mongolian gazelles using satellite-based estimates of vegetation productivity. *Journal of Applied Ecology*, 45 (2), 649-658.

Munson, L., Terio, K.A., Kock, R., Mlengeya, T., Roelke, M. E., Dubovi, E., Packer, C. (2008). Climate extremes promote fatal co-infections during canine distemper epidemics in African lions. *PloS One*, 3 (6), e2545.

Murphy, D., Mumby, H.S., Henley, M.D. (2020). Age differences in the temporal stability of a male African elephant (*Loxodonta africana*) social network. *Behavioral Ecology*, 31 (1), 21-31.

Muteti D, J., Mwita, M., Ndambuki, S., Mareale, Kenana, L., Ngene, S.M. & Omondi, P.O. (2018) Amboseli, Kilimanjaro, Magadi, Natron (AWKMAN) cross border landscape total aerial count June 2018.

Mutinda, H.S. (1995). Assessment of reproductive status of free ranging female African elephants *(Loxodonta africana)* using non-invasive techniques. Doctoral Dissertation. University of Nairobi.

Mwamuye, M.M., Kariuki, E., Omondi, D., Kabii, J., Odongo, D., Masiga, D., Villinger, J. (2017). Novel *Rickettsia* and emergent tick-borne pathogens: A molecular survey of ticks and tick-borne pathogens in Shimba Hills National Reserve, Kenya. *Ticks and Tick-borne Diseases*, 8 (2), 208-218.

Mwiu S, Kiambi S, Bett A, Mukeka J, Nyaligu M, Ikime T, *et al*. (2017) Aerial Total Count of Elephants, Buffalo and Giraffe in the Mara ecosystem (May 2017).

Nadolny, R., Gaff, H., Carlsson, J., Gauthier, D. (2015). Comparative population genetics of two invading ticks: Evidence of the ecological mechanisms underlying tick range expansions. *Infection, Genetics and Evolution*, 35, 153-162.

Nakandé, A., Belem, A.M.G., Nianogo, A.J., Jost, C. (2007). Parasites gastro-intestinaux des éléphants dans la Réserve Partielle de Pama, Burkina Faso. *Pachyderm*, 42, 22-32.

Neitz, A.W., Viljoen, G.J., Bezuidenhout, J.D., Oberem, P.T., Visser, L., Vermeulen, N.M. (1986). Detection of *Cowdria ruminantium* antigen and antibody during the course of heartwater disease in sheep by means of an enzyme-linked immunosorbent assay. *The Onderstepoort Journal of Veterinary Research,* 53(4), 205-207.

Ngene, S., Lala, F., Nzisa, M., Kimitei, K., Mukeka, J., Kiambi, S., ... & Douglas-Hamilton, I. (2017). Aerial Total Count of

Elephants, Buffalo and Giraffe in the Tsavo-Mkomazi Ecosystem*(February 2017). *Kenya Wildlife Service (KWS) and Tanzania Wildlife Research Institute (TAWIRI), Arusha*

Ngene, S., Okello, M.M., Mukeka, J., Muya, S., Njumbi, S., Isiche, J. (2017 b). Home range sizes and space use of African elephants (*Loxodonta africana*) in the Southern Kenya and Northern Tanzania borderland landscape. *International Journal of Biodiversity and Conservation,* 9(1), 9-26. doi.org/10.5897/IJBC2016.1033.

Njumbi, S. (1993). Effects of poaching on the population structure of elephants in Meru National Park. *MPhil, Moi University*.

Nicholson, S.E., Farrar, T.J. (1994). The influence of soil type on the relationships between NDVI, rainfall, and soil moisture in semiarid Botswana. I. NDVI response to rainfall. *Remote Sensing of Environment,* 50 (2), 107-120.

Nijhof, A.M., Penzhorn, B.L., Lynen, G., Mollel, J.O., Morkel, P., Bekker, C.P., Jongejan, F. (2003). *Babesia bicornis* sp. nov. and *Theileria bicornis* sp. nov.: tick-borne parasites associated with mortality in the black rhinoceros (*Diceros bicornis*). *Journal of Clinical Microbiology,* 41(5), 2249-2254.

Nijhof, A.M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W.H., Lawrence, J. A., Jongejan, F. (2005). Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. *Journal of Clinical Microbiology,* 43(12), 5907-5911.

Norval, R.A.I., Colborne, J., Tannock, J., Mackenzie, P.K.I. (1980). The life cycle of *Amblyomma tholloni* Neumann, 1899 (Acarina: Ixodidae) under laboratory conditions. *Veterinary Parasitology*, 7(3), 255-263.

Norval, R.A.I. (1983). The ticks of Zimbabwe. VII. The genus *Amblyomma*. *Zimbabwe Veterinary Journal*, 14(1/4), 3-18.

Norval, R.A.I., Perry, B.D., Meltzer, M.I., Kruska, R.L., Booth, T.H. (1994). Factors affecting the distributions of the ticks *Amblyomma hebraeum* and *A. variegatum* in Zimbabwe: implications of reduced acaricide usage. *Experimental & Applied Acarology*, 18(7), 383-407.

Obanda, V., Iwaki, T., Mutinda, N.M., Gakuya, F. (2011). Gastrointestinal parasites and associated pathological lesions in starving free-ranging African elephants. *African Journal of Wildlife Research*, 41(2), 167-172.

Oberem, P. T., & Bezuidenhout, J. D. (1987). Heartwater in hosts other than domestic ruminants. *The Onderstepoort journal of veterinary research*, *54*(3), 271.

Ogden, N. (2013). Changing geographic ranges of ticks and tick-borne pathogens: drivers, mechanisms and consequences for pathogen diversity. *Frontiers in Cellular and Infection Microbiology*, *3*, 46.

Okello, J.B., Masembe, C., Rasmussen, H.B., Wittemyer, G., Omondi, P., Kahindi, O., Siegismund, H.R. (2008). Population genetic structure of savannah elephants in Kenya: conservation and management implications. *Journal of Heredity*, 99(5), 443-452.

Okino, T., Ushirogawa, H., Matoba, K., Hatsushika, R. (2007). A bibliographical study of human cases of hard tick (Acarina: Ixodidae) bites received abroad and found in Japan. *Kawasaki Medical Journal*, 33, 189-194.

Osakabe, M., Uesugi, R., Goka, K. (2009). Evolutionary aspects of acaricide-resistance development in spider mites. *Psyche*, 2009.

Otiende, M.Y., Kivata, M.W., Makumi, J.N., Mutinda, M.N., Okun, D., Kariuki, L., Alasaad, S. (2015). Epidemiology of *Theileria bicornis* among black and white rhinoceros metapopulation in Kenya. *BMC Veterinary Research*, 11(1), 4.

Osborn, F.V. (2004). The concept of home range in relation to elephants in Africa. *Pachyderm,* (37), 37-44.

Oura, C.A.L., Asiimwe, B.B., Weir, W., Lubega, G.W., Tait, A. (2005). Population genetic analysis and sub-structuring of *Theileria parva* in Uganda. *Molecular and Biochemical Parasitology*, 140 (2), 229-239.

Pandey, V.S., Chitate, F., Nyanzunda, T.M. (1993). Epidemiological observations on gastro-intestinal nematodes in communal land cattle from the High yield of Zimbabwe. *Veterinary Parasitology*, 51(1-2), 99-106.

Parola, P., Raoult, D. (2001). Ticks and tick borne bacterial diseases in humans: an emerging infectious threat. *Clinical Infectious Diseases*, 32 (6), 897-928.

Park, H.S., Lee, J.H., Jeong, E.J., Park, T.K., Kim, T.Y., Chae, J.S., Lee, S. H. (2005). Differentiation of Anaplasmataceae

through partial groEL gene analysis. *Microbiology and Immunology*, 49 (7), 655-662.

Parker, J.M., Goldenberg, S.Z., Letitiya, D., Wittemyer, G. (2020). Strongylid infection varies with age, sex, movement and social factors in wild African elephants. *Parasitology*, 147 (3), 348-359.

Paruelo, J.M., Epstein, H.E., Lauenroth, W.K., Burke, I. C. (1997). ANPP estimates from NDVI for the central grassland region of the United States. *Ecology*, 78 (3), 953-958.

Patz, J.A., Graczyk, T.K., Geller, N., Vittor, A.Y. (2000). Effects of environmental change on emerging parasitic diseases. *International Journal for Parasitology*, 30 (12-13), 1395-1405.

Paulauskas, A., Galdikaitė-Brazienė, E., Radzijevskaja, J., Aleksandravičienė, A., Galdikas, M. (2016). Genetic diversity of *Ixodes ricinus* (Ixodida: Ixodidae) ticks in sympatric and allopatric zones in Baltic countries. *Journal of Vector Ecology*, 41 (2), 244-253.

Penzhorn, B.L. (2006). Babesiosis of wild carnivores and ungulates. *Veterinary Parasitology*, 138 (1-2), 11-21.

Perreau, P., Morel, P.C., Barré, N., Durand, P.Y. (1980). Existence de la cowdriose (heartwater) à *Cowdria ruminantium* chez les ruminants des Antilles françaises (La Guadeloupe) et des Mascareignes (La Réunion et Ile Maurice). *Revue d'élevage et de Médecine Vétérinaire des Pays Tropicaux*, 33 (1), 21-22.

Peter, T.F., Burridge, M.J., Mahan, S.M. (2002). *Ehrlichia ruminantium* infection (heartwater) in wild animals. *Trends in Parasitology*, 18 (5), 214-218.

Petney, T., Rechav, Y., Horak, I.G. (1987). The ecology of the African vectors of heartwater, with particular reference to *Amblyomma hebraeum* and *Amblyomma variegatum*. *Onderstepoort Journal of Veterinary Research,* (54).

Pfukenyi, D.M., Mukaratirwa, S. (2013). A review of the epidemiology and control of gastrointestinal nematode infections in cattle in Zimbabwe. *Onderstepoort Journal of Veterinary Research,* 80 (1), 01-12.

Piana, G.P., Galli-Valerio, B. (1895). Su di un'infezione del cane con parassiti endoglobulari nel sangue. *Nota Prev Mod Zooiatro,* 6, 163-9.

Poche, F. (1913). The system of the protozoa. *Arch. Protistenkunde,* 30 (3), 125-321.

Poole, J. H. (1982). *Musth and male-male competition in the African elepha*nt. Doctoral Dissertation. University of Cambridge.

Poole, J.H. (1987). Rutting behavior in African elephants: the phenomenon of musth. *Behaviour,* 102 (3-4), 283-316.

Poole, J. H., Payne, K., Langbauer, W. R., & Moss, C. J. (1988). The social contexts of some very low frequency calls of African elephants. *Behavioral Ecology and Sociobiology*, *22*(6), 385-392.

Poole, J.H. (1989 a). Announcing intent: the aggressive state of musth in African elephants. *Animal Behaviour,* 37, 140-152.

Poole, J.H. (1989 b). Mate guarding, reproductive success and female choice in African elephants. *Animal Behaviour*, 37, 842-849.

Poole, J.H. (1989 c). The effects of poaching on the age structure and social and reproductive patterns of selected East African elephant populations. *The Ivory Trade and the Future of the African elephant*, 2, 1-73.

Poole, J.H. (1994). Sex differences in the behaviour of African elephants. The differences between the sexes, *Cambridge University Press***,** 331-346.

Poole, J. H. (1999). Signals and assessment in African elephants: evidence from playback experiments. *Animal Behaviour*, *58*(1), 185-193.

Poole, J.H., Moss, C.J. (1981). Musth in the African elephant, *Loxodonta africana*. *Nature*, 292 (5826), 830-831.

Poole, J.H., Moss, C.J. (1989). Elephant mate searching: Group dynamics and vocal and olfactory communication. *Symposium Zoological Society of London*, 61,111-125.

Poole, J.H., Payne, K., Langbauer, W.R., Moss, C.J. (1988). The social contexts of some very low frequency calls of African elephants. *Behavioral Ecology and Sociobiology***,** 22 (6), 385-392.

Poole, J.H., Lee, P.C., Njiraini, N., Moss, C.J. (2011). Longevity, competition and musth: a long-term perspective on male reproductive strategies. *The Amboseli Elephants*, 272-290.

Prince, S.D. (1991). Satellite remote sensing of primary production: comparison of results for Sahelian grasslands 1981-

1988. *International Journal of Remote Sensing*, 12 (6), 1301-1311.

Prozesky, L., Bezuidenhout, J.D., Paterson, C.L. (1986). Heartwater: an in vitro study of the ultrastructure of *Cowdria ruminatium*. *The Onderstepoort Journal of Veterinary Research*, 53 (3), 153-159.

Ramírez-Soriano, A., Ramos-Onsins, S.E., Rozas, J., Calafell, F., Navarro, A. (2008). Statistical power analysis of neutrality tests under demographic expansions, contractions and bottlenecks with recombination. *Genetics*, 179 (1), 555-567.

Ramos-Onsins, S.E., Rozas, J. (2002). Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, 19 (12), 2092-2100.

Rasmussen, H.B. (2005). *Reproductive tactics of male African savannah elephants (Loxodonta africana)*. Doctoral Dissertation. University of Oxford.

Rasmussen, L.E., Schmidt, M.J., Henneous, R., Groves, D., Daves, G.D. (1982). Asian bull elephants: flehmen-like responses to extractable components in female elephant estrous urine. *Science*, 217 (4555), 159-162.

Rasmussen, L.E.L., Lee, T.D., Roelofs, W.L., Zhang, A., Daves, G. D. (1996). Insect pheromone in elephants. *Nature*, 379 (6567), 684-684.

Rasmussen, H.B., Okello, J.B.A., Wittemyer, G., Siegismund, H.R., Arctander, P., Vollrath, F., Douglas-Hamilton, I. (2008). Age- and tactic-related paternity success in male African elephants. *Behavioral Ecology*, 19 (1), 9-15.

Rees, J. L. (2003). Genetics of hair and skin color. *Annual review of genetics*, *37*(1), 67-90.

Remesar, S., Díaz, P., Prieto, A., Markina, F,, Díaz-Cao, J.M., López-Lorenzo, G., Fernández, G., López, C.M., Panadero, R., Díez-Baños, P., Morrondo, P. (2019). *Prevalence and distribution of Babesia* and *Theileria* species in roe deer from Spain. *International Journal Parasitology Parasites Wildlife.* 21, (9) :195-201.

Remesar, S., Díaz, P., Prieto, A., García-Dios, D., Fernández, G., López, C.M., Panadero, R., Díez-Baños, P., Morrondo, P. (2020). Prevalence and molecular characterization of *Anaplasma phagocytophilum* in roe deer (*Capreolus capreolus*) from Spain. *Ticks and Tick-borne Diseases*, 11 (2):101351.

Ristic, M. (1988). Babesiosis of domestic animals and man.

Robbertse, L., Baron, S., van der Merwe, N.A., Madder, M., Stoltsz, W.H., Maritz-Olivier, C. (2016). Genetic diversity, acaricide resistance status and evolutionary potential of a *Rhipicephalus microplus* population from a disease-controlled cattle farming area in South Africa. *Ticks and Tick-borne Diseases*, 7 (4), 595-603.

Rodhain, J., & Van Den Berghe, L. (1936). Contribution à l'étude des plasmodiums des singes africains. *Ann Soc Belg Med Trop*, *16*, 237-253.

Rondelli, M.T. (1926). Alcuni Ixodidi della Somalia Italiana. *Research Biology*, *1*, 33-43.

Rozas, J., Ferrer-Mata, A., Sánchez-Del Barrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E., Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution*, 34 (12), 3299-3302.

Rudzinska, M., Spielman, A., Lewengrub, S., Piesman, J., Karakashian, S. (1984). The sequence of developmental events of *Babesia microti* in the gut of *Ixodes dammini*. *Protistologica (Paris. 1965)*, 20 (4), 649-663.

Ryan, S.J., Cross, P.C., Winnie, J., Hay, C., Bowers, J., Getz, W. M. (2012). The utility of normalized difference vegetation index for predicting African buffalo forage quality. *The Journal of Wildlife Management*, 76 (7), 1499-1508.

Schalk, G., Forbes, M. R. (1997). Male biases in parasitism of mammals: effects of study type, host age, and parasite taxon. *Oikos*, 67-74.

Schnittger, L., Rodriguez, A.E., Florin-Christensen, M., Morrison, D.A. (2012). *Babesia*: a world emerging. *Infection, Genetics and Evolution*, 12 (8), 1788-1809.

Scrucca, L., Fop, M., Murphy, T.B., Raftery, A.E. (2016). Mclust 5: clustering, classification and density estimation using Gaussian finite mixture models. *The R Journal*, 8 (1), 289.

Seivwright, L.J., Redpath, S.M., Mougeot, F., Watt, L., Hudson, P.J. (2004). Faecal egg counts provide a reliable measure of *Trichostrongylus tenuis* intensities in free-living red grouse *Lagopus lagopus scoticus*. *Journal of Helminthology*, 78 (1), 69.

Shannon, G., Page, B., Slotow, R., Duffy, K. (2006). African elephant home range and habitat selection in Pongola Game Reserve, South Africa. *African Zoology***,** 41 (1), 37-44.

Sherrard-Smith, E., Chadwick, E.A., Cable, J. (2013). Climatic variables are associated with the prevalence of biliary trematodes in otters. *International Journal for Parasitology*, 43 (9), 729-737.

Sikes, S.K. (1971). *Natural history of the African elephant.* The Trinity Press.

Simonsen, K.L., Churchill, G.A., Aquadro, C.F. (1995). Properties of statistical tests of neutrality for DNA polymorphism data. *Genetics*, 141 (1), 413-429.

Smith, T., & Kilborne, F. L. (1893). *Investigations into the nature, causation, and prevention of Texas or southern cattle fever* (No. 1). US Department of Agriculture, Bureau of Animal Industry.

Smith, R.M., Bhoora, R.V., Kotzé, A., Grobler, J.P., Dalton, D.L. (2019). Translocation a potential corridor for equine piroplasms in Cape mountain zebra (*Equus zebra zebra). International Journal for Parasitology: Parasites and Wildlife*, 9, 130-133.

Soulsby, E. J. L. (1982). Helminths, arthropods and protozoa of domesticated animals. *Helminths, arthropods and protozoa of domesticated animals.*, (Ed. 7).

Sousa, W.P., Grosholz, E.D. (1991). *The influence of habitat structure on the transmission of parasites*. In: *Habitat Structure*, pp. 300-324. Springer, Dordrecht.

Starcovici, C. (1893). Bemerkungen über den durch Babes entdeckten Blutparasiten und die durch denselben hervorgebrachten Krankheiten, die seuchenhafte Hämoglobinurie des Rindes (Babes), das Texasfieber (Th. Smith) und der Carceag der Schafe (Babes). *Zbl. Bakt., I. Abt*, *14*, 1-8.

Stucky, B.J. (2012). SeqTrace: a graphical tool for rapidly processing DNA sequencing chromatograms. *Journal of Biomolecular Techniques: JBT*, 23(3), 90.

Sukumar, R. (2003). *The living elephants: evolutionary ecology, behaviour, and conservation*. Oxford University Press.

Swai, E.S., Karimuribo, E.D., Rugaimukamu, E.A., Kambarage, D.M. (2006). Factors influencing the distribution of questing ticks and the prevalence stimation of *T. parva* infection in brown ear ticks in the Tanga region, Tanzania. *Journal of Vector Ecology***,** 31 (2), 224-228.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123(3), 585-595.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30 (12), 2725-2729.

Taylor, L. A., Vollrath, F., Lambert, B., Lunn, D., Douglas-Hamilton, I., Wittemyer, G. (2019). Movement reveals reproductive tactics in male elephants. *Journal of Animal Ecology*, 89 (1), 57-67.

Team, R. C. (2019). R: A language and environment for statistical computing (Version 3.0. 2) [Computer software]. Vienna, Austria: R Foundation for Statistical Computing.

Team, R. C. (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing Vienna Austria. 2018; version 3.5. 1.

Teshale, S., Geysen, D., Ameni, G., Asfaw, Y., Berkvens, D. (2015). Improved molecular detection of *Ehrlichia* and *Anaplasma* species applied to *Amblyomma* ticks collected from cattle and sheep in Ethiopia. *Ticks and Tick-borne Diseases,* 6 (1),1-7.

Theiler, G. (1962). The Ixodoidea parasites of vertebrates in Africa south of the Sahara (Ethiopian region). *Report of the Director of Veterinary Services-Onderstepoort.* Project 59958.

Thielsch, A., Knell, A., Mohammadyari, A., Petrusek, A., & Schwenk, K. (2017). Divergent clades or cryptic species? Mito-nuclear discordance in a Daphnia species complex. *BMC Evolutionary Biology*, *17*(1), 227.

Thomas, B., Holland, J.D., Minot, E.O. (2008). Elephant (*Loxodonta africana*) home ranges in Sabi Sand Reserve and Kruger National Park: a five-year satellite tracking study. *PLoS One,* 3 (12), e3902.

Thouless, C.R., Sakwa, J. (1995). Shocking elephants: fences and crop raiders in Laikipia District, Kenya. *Biological Conservation***,** 72(1),99-107.

Thurber, M.I., O'Connell-Rodwell, C.E., Turner, W.C., Nambandi, K., Kinzley, C., Rodwell, T. C., Bouley, D.M. (2011). Effects

of rainfall, host demography, and musth on strongyle fecal egg counts in African elephants (*Loxodonta africana*) in Namibia. *Journal of Wildlife Diseases,* 47 (1), 172-181.

Tucker, C.J., Vanpraet, C.L., Sharman, M.J., Van Ittersum, G. (1985). Satellite remote sensing of total herbaceous biomass production in the Senegalese Sahel: 1980–1984. *Remote Sensing of Environment,* 17 (3), 233-249.

Turner, W.C., Getz, W.M. (2010). Seasonal and demographic factors influencing gastrointestinal parasitism in ungulates of Etosha National Park. *Journal of Wildlife Diseases,* 46 (4), 1108-1119.

Uilenberg, G. (1983). Heartwater (*Cowdria ruminantium* infection): current status. *Advances in Veterinary Science and Comparative Medicine,* 27, 427-480.

Uilenberg, G. (2006). *Babesia*—a historical overview. *Veterinary Parasitology,* 138 (1-2), 3-10.

Uilenberg, G., Estrada-Peña, A., Thal, J. (2013). Ticks of the Central African Republic. *Experimental and Applied Acarology,* 60 (1), 1-40.

Vachiéry, N., Jeffery, H., Pegram, R., Aprelon, R., Pinarello, V., Kandassamy, R.L.Y., Frebling, M. (2008). *Amblyomma variegatum* ticks and heartwater on three Caribbean Islands: Tick infection and *Ehrlichia ruminantium* genetic diversity in bovine herds. *Annals of the New York Academy of Sciences,* 1149 (1), 191-195.

Van Der Westhuysen, O.P. (1938). A monograph on the helminth parasites of the elephant. *Onderstepoort Journal of Veterinary Science and Animal Industry*, 10 (1), 49-190.

Vander Waal, K., Omondi, G.P., Obanda, V. (2014). Mixed-host aggregations and helminth parasite sharing in an East African wildlife–livestock system. *Veterinary Parasitology*, 205 (1-2), 224-232.

Vázquez-López, M., Pego-Reigosa, R., Díez-Morrondo, C., Castro-Gago, M., Díaz, P., Fernández, G., Morrondo, P. (2015). Epidemiology of Lyme disease in a healthcare area in north-west Spain. *Gaceta Sanitaria*, 29(3), 213-216.

Vidya, T.N.C., Sukumar, R. (2002). The effect of some ecological factors on the intestinal parasite loads of the Asian elephant (*Elephas maximus*) in southern India. *Journal of Biosciences*, 27 (5), 521-528.

Vudriko, P., Okwee-Acai, J., Tayebwa, D.S., Byaruhanga, J., Kakooza, S., Wampande, E., Hatta, T. (2016). Emergence of multi-acaricide resistant *Rhipicephalus* ticks and its implication on chemical tick control in Uganda. *Parasites & Vectors*, 9 (1), 4.

Walker, J.B. (1957). *Rhipicephalus humeralis* Rondelli 1926. *Parasitology*, 47 (1-2), 145-152.

Walker, J.B., Olwage, A. (1987). The tick vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, genus Amblyomma) and their distribution. *Onderstepoort Journal of Veterinary Research*, 54.

Walker, J.B., Keirans, J.E., Horak, I.G. (2005). *The genus Rhipicephalus (Acari, Ixodidae): a guide to the brown ticks of the world.* Cambridge University Press.

Watt, D., Kiara, H., Sparagano, O.A.E. (1998). A PCR-based Field Evaluation of Theileria Infections in Cattle and Ticks in Kenya**.** *Annals of the New York Academy of Sciences*, 849 (1), 69-77.

Wenyon, C.M. (1926). *Protozoology. A Manual for Medical Men, Veterinarians and Zoologists.* Vol. 2.

Wesonga, F.D., Mukolwe, S.W., Grootenhuis, J. (2001). Transmission of *Cowdria ruminantium* by *Amblyomma gemma* from infected African buffalo (*Syncerus caffer*) and eland (*Taurotragus oryx*) to sheep. *Tropical Animal Health and Production*, 33 (5), 379-390.

Western, D. (1986). An odyssey to save the African elephant. *Discover Magazine.*

Western, D. (1989). The ecological role of elephants in Africa. *Discover Magazine.*

Western, D., Lindsay, W.K. (1984). Seasonal herd dynamics of a savanna elephant population. *African Journal of Ecology*, 22 (4), 229-244.

White, L.J., Tutin, C.E., Fernandez, M. (1993). Group composition and diet of forest elephants, *Loxodonta africana cyclotis* Matschie 1900, in the Lopé Reserve, Gabon. *African Journal of Ecology*, 31 (3), 181-199.

Whitmore Jr,F.C. (1977). Maglio, VJ Origin and evolution of the elephantidae. *Transactions of the American Philosophical Society, New Series*, 63, 1-149.

Wilson, D.E., Hirst, S.M. (1977). Ecology and factors limiting roan and sable antelope populations in South Africa. *Wildlife Monographs*, (54), 3-111.

Wilson, L. B., & Chowning, W. M. (1904). Studies in Pyroplasmosis Hominis. ("Spotted Fever" Or "Tick Fever" Of The Rocky Mountains.)(With Map, Charts, And Plates I, Ii). *Journal of Infectious Diseases*, *1*(1), 31-57.

Wilson, D.E., Bartsch, R.C., Bigalke, R.D., Thomas, S.E. (1974). Observations on mortality rates and disease in roan and sable antelope on nature reserves in the Transvaal. *South African Journal of Wildlife Research-24-month delayed open access*, 4 (3), 203-206.

Wittemyer, G., Douglas-Hamilton, I., Getz, W.M. (2005). The socioecological of elephants: analysis of the processes creating multitiered social structures. *Animal Behaviour*, 69 (6), 1357-1371.

Wittemyer, G., Northrup, J.M., Bastille-Rousseau, G. (2019). Behavioural valuation of landscapes using movement data. *Philosophical Transactions of the Royal Society B*, 374 (1781), 20180046.

Wright, S. (1949). The genetical structure of populations. *Annals of Eugenics*, 15 (1), 323-354.

Yusufmia, S.B.A.S., Collins, N.E., Nkuna, R., Troskie, M., Van den Bossche, P., Penzhorna, B. L. (2010). Occurrence of *Theileria*

parva and other haemoprotozoa in cattle at the edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. *Journal of the South African Veterinary Association*, 81 (1), 45-49.

Zimmerman, D.E. (2010). *The occurrence of piroplasms in various South African black rhinoceros (Diceros bicornis) populations.* Doctoral Dissertation. University of Pretoria.

Zuk, M., McKean, K.A. (1996). Sex differences in parasite infections: patterns and processes. *International Journal for Parasitology*, 26 (10), 1009-1024.